10

AUSTRALIAN JOURNAL
OF
BIOLOGICAL SCIENCES

VOLUME 12

MELBOURNE
1959

AUSTRALIAN JOURNAL OF BIOLOGICAL SCIENCES

A medium for the publication of results of original scientific research in the biological sciences with special emphasis on the experimental phases. Volumes 1 to 5 issued as the Australian Journal of Scientific Research, Series B: Biological Sciences.

Published by the Commonwealth Scientific and Industrial Research Organization. Issued quarterly, £2 per annum.

BOARD OF STANDARDS

The Board of Standards for this Journal is appointed by the Commonwealth Scientific and Industrial Research Organization and the Australian Academy of Science and consists of Professor J. G. Wood (Chairman), Dr. N. S. Noble (Editor), Professor J. S. Anderson, Professor Sir Macfarlane Burnet, Professor Sir Leslie Martin, and Professor W. P. Rogers.

ADVISORY COMMITTEE

Acceptance of papers for this Journal is in the hands of an Advisory Committee appointed by the Board of Standards and consisting of Dr. N. S. Noble (Chairman and Editor), Professor Sir Macfarlane Burnet, Dr. R. N. Robertson, Professor W. P. Rogers, and Professor J. G. Wood.

◆

All enquiries and manuscripts should be forwarded to:
The Editor,
Australian Journal of Biological Sciences,
Commonwealth Scientific and Industrial Research Organization,
314 Albert Street, East Melbourne, C.2, Victoria

MELBOURNE

CONTENTS

Number 1, February 1959

PAGE

The Genetic Effects of Family Structure in Natural Populations. By P. A. P. Moran and G. A. Watterson 1

Uptake and Translocation of Chlorine in Citrus Cuttings during and after a Short Salt Treatment. By H. Groenewegen, D. Bouma, and C. T. Gates 16

Calcium in the Nitrogen Metabolism of Subterranean Clover. By J. F. Loneragan 26

Physiology of Cereal Grain. I. The Source of Carbon for the Developing Barley Kernel. By M. S. Buttrose and L. H. May 40

Formation of the Colony in the Fungus *Chaetomium*. By N. J. B. Plomley .. 53

Strain Differences in the Morphology of the Gametes of the Mouse. By A. W. H. Braden 65

The Pre-natal Development of Skin and Hair in Cattle (*Bos taurus* L.). By A. G. Lyne and Margaret J. Heideman 72

The Electrophoretic Properties of *S*-Carboxymethylkerateines and *S*-Sulphokerateines from Wool. By E. F. Woods 96

Number 2, May 1959

PAGE

The Theory of some Genetical Effects of Population Subdivision. By P. A. P. Moran 109

A Study of High Temperature Lesions in *Arabidopsis thaliana*. By J. Langridge and B. Griffing 117

The Water Permeability of Cells of *Chara australis* R. Br. By. J Dainty and A. B. Hope 136

Physiology of Cereal Grain. II. Starch Granule Formation in the Developing Barley Kernel. By L. H. May and M. S. Buttrose 146

The Effect of Chloride on the Free Amino Acids of Cabbage and Cauliflower Plants. By J. R. Freney, C. C. Delwiche, and C. M. Johnson 160

Oxidation of Krebs Cycle Acids by Tissue Slices and Cytoplasmic Particles from Apple Fruit. By M. D. Hatch, Judith A. Pearson, Adele Millerd, and R. N. Robertson 167

Studies in the Natural Coating of Apples. IV. The Nature of Cutin. By F. E. Huelin 175

A DPNH-specific Nitrate Reductase from Germinating Wheat. By D. Spencer 181

Formation of a Polysaccharide in Sugar-cane. By R. I. Nicholson and B. Lilienthal 192

The Adsorption by *Aerobacter aerogenes* of *p*-Hydroxybenzoic Acid and its Esters and of Sodium Benzoate at Different pH Levels. By R. G. H. Barbour 204

Some Comparative Properties of β-Glucosidases Secreted by Fungi. By M. A. Jermyn 213

NUMBER 3, AUGUST 1959

PAGE

Studies on Plant Pathogenic Bacteria. I. Cultural and Biochemical Characters. By Rose Mushin, Jill Naylor, and N. Lahovary 223

Studies on Plant Pathogenic Bacteria. II. Serology. By Rose Mushin, Jill Naylor, and N. Lahovary 233

The Influence of the Soil Moisture Regime on the Expulsion of the Larval Mass of the Nematode *Anguina agrostis* from Galls. By N. Collis-George and C. D. Blake 247

Extension-growth Activities of some *cyclo*Propane Derivatives, a New Class of Antiauxin. By N. P. Kefford 257

Studies on "Haemixodovin", the Pigment in the Eggs of the Cattle Tick *Boophilus microplus* (Acarina: Ixodidae). By K. C. Bremner 263

The Digestion of Wool by a Keratinolytic Bacillus. By G. S. Molyneux .. 274

Studies on Oxidized Wool. I. A Comparison of the Completeness of Oxidation with Peracetic and Performic Acids. By E. O. P. Thompson and I. J. O'Donnell 282

Studies on Oxidized Wool. II. Extraction of Soluble Proteins from Wool Oxidized with Performic Acid. By I. J. O'Donnell and E. O. P. Thompson 294

A Method for the Isolation of DNP-Cysteic Acid and its Application to the Determination of *N*-Terminal Half-cystine Residues in Wool. By E. O. P. Thompson 303

The Development and Growth of Vibrissae in the House Mouse with Particular Reference to the Time of Action of the Tabby (*Ta*) and Ragged (*Ra*) Genes. By R. B. Dun 312

Variation of Vibrissa Number in the Mouse. By A. S. Fraser, T. Nay, and B. Kindred 331

Short Communications

Neutral and Acidic Auxins in Developing Tobacco Fruit. By L. G. Paleg and R. M. Muir 340

Differential Specificity Exhibited by Two Germination Inhibitors Present in *Echium plantagineum* L. By L. A. T. Ballard and A. E. Grant Lipp .. 343

Number 4, November 1959

PAGE

Translocation of Labelled Assimilates in the Soybean. By R. Thaine, Stella L. Ovenden, and J. S. Turner 349

Residual Effects of Auxin, Chelating Agents, and Metabolic Inhibitors in Cell Extension. By D. J. Carr and E. K. Ng 373

Potentiation of Lateral Root Induction by Root Initials in Isolated Flax Roots. By P. L. Goldacre 388

Ionic Relations of Cells of *Chara australis*. I. Ion Exchange in the Cell Wall. By J. Dainty and A. B. Hope 395

Studies of Action Potentials in the Vacuole and Cytoplasm of *Nitella*. By G. P. Findlay 412

Nodulation Studies in Legumes. III. The Effects of Delaying Inoculation on the Seedling Symbiosis of Barrel Medic, *Medicago tribuloides* Desr. By P. J. Dart and J. S. Pate 427

Chemical Changes during Growth and Storage of the Avocado Fruit. By J. B. Davenport and S. C. Ellis 445

Hypothyroidism in the Sheep. By A. K. Lascelles and B. P. Setchell .. 455

Some Effects of Fluoride on Calcium Metabolism in the Bones of Young Rats. By J. R. Dunstone and E. Payne 466

Studies of Casein. V. The Action of Rennin on Casein. By R. G. Wake .. 479

Studies on Oxidized Wool. III. Ion-exchange and Acid-uptake Characteristics. By E. O. P. Thompson and I. J. O'Donnell 490

Genetic Sampling in a Random Mating Population of Constant Size and Sex Ratio. By B. D. H. Latter 500

Selection for an Invariant Character, Vibrissa Number, in the House Mouse. By R. B. Dun and A. S. Fraser 506

Variation and Dominance at the Scute Locus in *Drosophila melanogaster*. By J. M. Rendel 524

Short Communications

The Use of Thyroxine in the Bioassay of Growth Hormone. By A. L. Wallace 534

Studies of Casein. IV. The Isolation of κ-Casein. By R. G. Wake 538

Index to Volume 12 541

THE GENETIC EFFECTS OF FAMILY STRUCTURE IN NATURAL POPULATIONS

By P. A. P. Moran* and G. A. Watterson*

[*Manuscript received August* 27, 1958]

Summary

Every finite interbreeding genetic population not subject to gene mutation will eventually become homozygous. In this paper, the rate at which this state is reached is found for a dioecious diploid population whose generations do not overlap. The effect of family structure on this rate is investigated, and it is shown that the rate for a randomly mating population remains unchanged if there is permanent marriage (monogamy) amongst individuals. The determining factors are shown to be the variances of the number of offspring per individual of specified sex, and therefore the probability distributions of offspring are studied for several population models, and, in particular, are related to the birth and death rates for each individual of specified age.

Although the general solution for overlapping generation models has not been found, it is shown that one particular model behaves the same as the equivalent non-overlapping generation model. But this result does not hold in general, as is shown by a second pair of equivalent models in which the nature of the generations does affect the asymptotic behaviour. The conditions under which the asymptotic behaviour of an overlapping model may be inferred from that of a non-overlapping model are not known.

I. Introduction

Any finite genetic population not subject to mutation will eventually reach an absorbing state in which all individuals are of the same homozygous type. The probability that the population has not reached an absorbing state decreases to zero with increasing generations, and at the tth generation ($t = 0, 1, 2, \ldots$) the probability will be asymptotically equal to an expression of the form $C\lambda^t$, where C is a constant and λ is called the "rate of approach to homozygosity".

It is to be expected that the actual value taken by λ will depend on the specific population model being considered, and in fact we shall find that one factor of importance is the manner in which the offspring of one generation are distributed among the parents. The only general model of this kind that has been investigated is one for a monoecious population. Consider such a population of N diploid individuals, and write σ^2 as the variance of the number of diploid offspring from a single parent. By relating this model to that of a population with $2N$ haploid individuals, Wright has shown that the "effective population size" is

---

*Department of Statistics, Australian National University, Canberra.

$(4N-2)/(\sigma^2+2)$ which is equivalent in our terminology to saying that the asymptotic rate of approach to homozygosity is

$$\lambda = 1-\{(\sigma^2+2)/(8N-4)\}.$$

A brief derivation of this result is given in Li (1955, p. 321), and it was also obtained by Haldane (1939) and Fisher (1939).

For dioecious populations of diploid individuals only particular examples have been studied. Wright (1931, 1933) found that the rate of approach, for a population with N_1 males, and N_2 females practising a mating system to be described in the next section, is

$$\lambda \doteqdot 1-N/8N_1N_2,$$

for autosomal genes, where $N = N_1+N_2$, and for sex-linked genes it is

$$\lambda \doteqdot 1-(N_2+2N_1)/9N_1N_2.$$

In Wright's model, the generations are kept distinct and mating between them is not possible. An alternative model has been proposed for which the generations do overlap (Moran 1958), and the resulting rate of approach for autosomal genes is

$$\lambda \doteqdot 1-N/4N_1N_2.$$

Finally, a third model very similar to the previous one, and having overlapping generations, was shown (Watterson, unpublished data) to have a rate identical to Wright's model,

$$\lambda \doteqdot 1-N/8N_1N_2.$$

All these dioecious models have specific distributions for the number of offspring per individual of the parent generation, and it is our aim to generalize the results to cases with arbitrary offspring distributions, and also investigate the effect that permanent marriage between sexes has on the population's asymptotic behaviour. Therefore, we first consider populations from the point of view of family structure and size, and then introduce genetic considerations and investigate the effect of family structure on the asymptotic genetic behaviour of the population.

II. Family Structure

For a given dioecious population, let $\pi_1(x)$ be the probability of a male individual surviving to the age x at least, and $\pi_2(x)$ be the similar probability for a female individual. For these quantities to be well defined, it is implicitly assumed that no selective effects are present, and that all individuals of same sex are equivalent with respect to lifetime distributions. With the same assumption, we may define two further quantities $m_1(x)\mathrm{d}x$ and $m_2(x)\mathrm{d}x$ to be the probabilities that a male and female of age x will produce an offspring of either sex in the time interval $(x, x+\mathrm{d}x)$. We suppose that such events occur independently in different intervals of x, i.e. we ignore the effect of a finite length of pregnancy and suppose that the probability of producing an offspring at any given age is independent of what has happened before. Then the number of offspring produced by a male which lived to an age x and then died in the interval $(x, x+\mathrm{d}x)$, an event with probability $-\pi_1'(x)\,\mathrm{d}x$, has a Poisson distribution with mean

$$M_1(x) = \int_0^x m_1(t)\,\mathrm{d}t,$$

and a probability generating function

$$\exp\left\{(z-1)\int_0^x m_1(t)\,\mathrm{d}t\right\}.$$

Averaging over all possible ages we therefore get the probability generating function for the total number of offspring from a male individual :

$$P_1(z) = -\int_0^\infty \exp\left\{(z-1)\int_0^x m_1(t)\,\mathrm{d}t\right\}\pi_1'(x)\,\mathrm{d}x. \qquad \ldots\ldots.(\mathbf{1})$$

Similarly for offspring from a female individual the generating function is

$$P_2(z) = -\int_0^\infty \exp\left\{(z-1)\int_0^x m_2(t)\,\mathrm{d}t\right\}\pi_2'(x)\,\mathrm{d}x. \qquad \ldots\ldots.(\mathbf{2})$$

Such generating functions are to play a large part in the next section of the paper, so we shall consider them in somewhat more detail. In the above, we have made several simplifying assumptions which may or may not be valid when an actual population is investigated. Nevertheless, generating functions for the number of offspring per individual are important whether equations (**1**) and (**2**) are applicable or not. For example, if the generations of a population neither overlap nor interbreed, then a workable theoretical model can be formulated in which all individuals die simultaneously, and the entire succeeding generation is born at that instant. For this model the previous birth and death rate functions are degenerate, but of course the generating functions may be defined from first principles. Thus if the ith male has x_i offspring, and $Pr\{x_i = n\} = p_n$ (say), then the generating function is

$$P_1(z) = \sum_{n=0}^{\infty} p_n z^n.$$

The simplest way to introduce monogamy ("marriage") into a population model is to assume that the sex numbers are equal and allow permanent random pairing between members of opposite sex. One consequence of this pairing is that both parents in the family have the same number of offspring, and hence the generating functions $P_1(z)$ and $P_2(z)$ must be identical.

Typical examples of generating functions which may be of use in practical cases are those corresponding to Poisson, geometric, and binomial distributions for the x_i variates. The first two of these distributions allow the x_i to vary over all integers, whilst the latter restricts them to a finite range. Most theoretical models which have been found suitable for mathematical investigation restrict the population size to exactly N for all generations, the sex numbers being held constant at N_1 and N_2. For these models the offspring distributions must have finite range, but it is interesting to consider how such a model may approximate an actual population with arbitrary offspring distributions, perhaps of infinite range.

Let us concentrate on the male parents. Given any generating function $Q(z)$ for a random variate y taking integral values, the joint generating function for N_1 independent variables $y_1, y_2, \ldots, y_{N_1}$ (say) is

$$Q(z_1)\, Q(z_2) \ldots\ldots\ldots Q(z_{N_1}).$$

If the variable y_i is the number of offspring from the ith male in a population, then the next generation will have size Σy_i with expectation $N_1 Q'(1)$. To construct a constant size model approximating to this population, the numbers of offspring, per male, must be dependent random variables $x_1, x_2, \ldots, x_{N_1}$ (say) with total $\Sigma x_i = N$. Suppose the x_i have identical distributions with generating function $P_1(z)$ and write $p_n = Pr\{x_i = n\}$, $q_n = Pr\{y_i = n\}$. For the model to fit the actual population approximately, we need $N = N_1 P_1'(1) = N_1 Q'(1)$ and $p_n \doteqdot q_n$. In fact, we shall see that the former condition is sufficient to ensure the latter.

The joint generating function for the x_i variables (conditional on $\Sigma x_i = N$) is

$$\frac{\text{coefficient of } w^N \text{ in } Q(z_1 w)\, Q(z_2 w)\ \ldots\ Q(z_{N_1} w)}{\text{coefficient of } w^N \text{ in } Q(w)^{N_1}},$$

thus with $z = z_1$, $z_2 = z_3 = \ \ldots\ = z_{N_1} = 1$,

$$P_1(z) = \frac{\text{coefficient of } w^N \text{ in } Q(zw)\, Q(w)^{N_1}}{\text{coefficient of } w^N \text{ in } Q(w)^{N_1}},$$

and so

$$p_n = \text{coefficient of } z^n \text{ in } P_1(z),$$

$$= q_n \frac{\text{coefficient of } w^{N-n} \text{ in } Q(w)^{N_1-1}}{\text{coefficient of } w^N \text{ in } Q(w)^{N_1}}.$$

To prove that $p_n \to q_n$ as N_1, $N \to \infty$ provided $N = N_1 P_1'(1) = N_1 Q'(1)$, we must show that the coefficients of w^{N-n} in $Q(w)^{N_1-1}$ and of w^N in $Q(w)^{N_1}$ are asymptotically equal. This is obviously true if NN_1^{-1} is an integer, $n = NN_1^{-1}$, and $Q(w) = w^{NN_1^{-1}}$. Suppose that $Q(w)$ has a non-zero finite variance. Then $Q(w)^{N_1}$ is the generating function of a discrete probability distribution whose ordinates, by a form of the Central Limit Theorem, are asymptotically equal to quantities proportional to the ordinates of a normal distribution with mean N and variance proportional to N_1. The coefficient of w^N will tend to the central ordinate, and since n is fixed and tends to zero in comparison with the standard deviation, the coefficient of w^{N-n} in $Q(w)^{N_1-1}$ will also tend to the central ordinate. Thus under these conditions, $p_n \to q_n$, and this convergence is easily shown to be uniform on n. The correspondence between the two populations, however, only holds from one generation to the next, and clearly the constant size model will not have the same behaviour after many generations as another population which may die out completely.

As we have seen, for a population to remain at constant size, the means of the generating functions for each sex must be

$$P_1'(1) = NN_1^{-1}\,,\ P_2'(1) = NN_2^{-1}. \qquad \ldots\ldots\ldots\ldots(3)$$

Because the constraint imposes a degree of dependence between the numbers of offspring from two males (or females) there is a covariance c_1 (say) between those for the males, and similarly c_2 between females. We write σ_1^2 for the variance of the distribution defined by $P_1(z)$, and σ_2^2 for the distribution defined by $P_2(z)$. Now because $\sum_1^{N_1} x_i = N$, we must have $N_1\sigma_1^2+N_1(N_1-1)c_1 = 0$, so that

$$\left.\begin{aligned} c_1 &= -\sigma_1^2(N_1-1)^{-1} = -(P_1''(1)+P_1'(1)-P_1'(1)^2)(N_1-1)^{-1}, \\ &\text{and similarly} \\ c_2 &= -\sigma_2^2(N_2-1)^{-1} = -(P_2''(1)+P_2'(1)-P_2'(1)^2)(N_2-1)^{-1}. \end{aligned}\right\} \quad ..(4)$$

The first two moments of the offspring distribution are critical quantities for subsequent theory. For a population with non-degenerate birth and death rates, we have from (**1**) and (**2**) that

$$P_1'(1) = \int_0^\infty m_1(x)\, \pi_1(x)\, \mathrm{d}x,$$

$$P_2'(1) = \int_0^\infty m_2(x)\, \pi_2(x)\, \mathrm{d}x,$$

found by integrating by parts and noting that $\pi_i(x)$ tends to zero as x tends to infinity. For constant expected population size these means must equal NN_1^{-1} and NN_2^{-1} respectively. Similarly we find the second derivatives

$$P_1''(1) = 2\int_0^\infty m_1(x)\, \pi_1(x) \left\{\int_0^x m_1(t)\, \mathrm{d}t\right\} \mathrm{d}x = 2\int_0^\infty m_1(x)\, \pi_1(x)\, M_1(x)\, \mathrm{d}x,$$

$$P_2''(1) = 2\int_0^\infty m_2(x)\, \pi_2(x) \left\{\int_0^x m_2(t)\, \mathrm{d}t\right\} \mathrm{d}x = 2\int_0^\infty m_2(x)\, \pi_2(x)\, M_2(x)\, \mathrm{d}x.$$

One other aspect of this problem deserves mention (see Fisher 1939). Consider a population in which the generations do not overlap and for which each individual has a large number of offspring only a few of which survive. Then the model we use will depend on the stage at which the population is enumerated. If the population of sexually mature adults is of size N, and for simplicity the sex numbers are equal, $N_1 = N_2 = \frac{1}{2}N$, and if each individual has a probability distribution of producing immature individuals with generating function $R(z)$, then the correct generating functions to use in the model will be

$$P_1(z) = P_2(z) = R(1-\pi+\pi z), \quad ....(5)$$

where π is the probability of an immature individual surviving to maturity. If on the other hand we enumerate the immature individuals the correct generating functions to use will be

$$P_1(z) = P_2(z) = 1-\pi+\pi R(z), \quad ....(6)$$

together with the much larger population size $\frac{1}{2}NR'(1)$. Since the genetic results must be the same we have set up an interesting equivalence between populations of different size and different distributions of the numbers of offspring. As a simple example, the expected population size will be constant only if

$$P_1'(1) = \pi R'(1) = N(\tfrac{1}{2}N)^{-1} = 2,$$

in the first case, and in the second if

$$P_1'(1) = \pi R'(1) = \tfrac{1}{2}NR'(1)\{\tfrac{1}{4}NR'(1)\}^{-1} = 2.$$

Both conditions are satisfied if $R'(1) = 2\,\pi^{-1}$.

To conclude this section, some examples will be given of generating functions applying to population models previously studied. Perhaps the most important is the following.

(*a*) *Wright's Model*

Wright (1931) studied a population having non-overlappping generations, and assumed that N offspring resulted from matings formed by random sampling, with replacement, amongst the N_1 male and N_2 female parents. Thus the number of offspring per individual is a binomial variate, and the generating functions are

$$\left.\begin{aligned} P_1(z) &= (1-N_1^{-1}+N_1^{-1}z)^N, \\ P_2(z) &= (1-N_2^{-1}+N_2^{-1}z)^N. \end{aligned}\right\} \quad \ldots\ldots\ldots\ldots\ (7)$$

(*b*) *Degenerate Model*

If all male individuals have exactly NN_1^{-1} offspring, all females exactly NN_2^{-1}, these being integers, then the variances σ_1^2 and σ_2^2 are both zero, and the generating functions are

$$\left.\begin{aligned} P_1(z) &= z^{NN_1^{-1}}, \\ P_2(z) &= z^{NN_2^{-1}}. \end{aligned}\right\} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (8)$$

(*c*) *Overlapping Generation Model* I

In a previous paper (Moran 1958) a model has been constructed in which the generations are overlapping. Individuals die at random and are replaced by new individuals formed by the mating of gametes chosen from the output of the population before the death took place. The probability of any given individual dying at such a birth-death event is N^{-1}, and of having a life time of exactly n units is $N^{-1}(1-N^{-1})^{n-1}$, $n = 1, 2, 3, \ldots$. This may be expressed by saying that $n-1$ has a geometric distribution; the expected life time is N and is the number of time units corresponding to one generation in a non-overlapping model.

At each instant at which a death occurs, including its own, the probability of the individual becoming a parent is N_1^{-1} if male, N_2^{-1} if female, so that the probability generating function of the number of offspring per male is

$$\begin{aligned} P_1(z) &= \sum_{n=1}^{\infty} N^{-1}(1-N^{-1})^{n-1}(1-N_1^{-1}+N_1^{-1}z)^n \\ &= (N_1-1+z)\{N+N_1-1-(N-1)z\}^{-1}, \end{aligned}$$

and similarly for the females

$$P_2(z) = (N_2-1+z)\{N+N_2-1-(N-1)z\}^{-1}. \quad \ldots (9)$$

(d) *Overlapping Generation Model* II

Watterson (unpublished data) has considered an overlapping model slightly different from that above by assuming that individuals die at random, and each is replaced by a new individual with one parent being the dying individual, the other being chosen at random from the opposite sex. In this case the probability of an individual living for exactly n time units is $N^{-1}(1-N^{-1})^{n-1}$, $n = 1, 2, 3, \ldots$, as before, but the probability of its being a parent at any stage is altered. It must always be a parent at its own death. Consider an event not involving its death, but being the death of one of the $N-1$ remaining individuals. If the original individual was male, then for it to be a parent at a death other than its own, the dying individual must be of opposite sex (probability $N_2(N-1)^{-1}$) and the original individual must be chosen as the second parent from N_1 similar males. Therefore the required probability is $N_2(N-1)^{-1}N_1^{-1}$, and the generating function becomes

$$P_1(z) = \sum_{n=1}^{\infty} N^{-1}(1-N^{-1})^{n-1}\{1-N_2(N-1)^{-1}N_1^{-1}+N_2(N-1)^{-1}N_1^{-1}z\}^{n-1}z,$$

$$\left.\begin{aligned} &= zN_1(N-N_2z)^{-1}. \\ &\text{Similarly} \\ P_2(z) &= zN_2(N-N_1z)^{-1}. \end{aligned}\right\} \quad \ldots\ldots\ldots\ldots\ldots\ldots (10)$$

The latter two models have generating functions closely akin to those for geometrically distributed variates. Model I differs from this by altering the probability of an individual having no offspring, whilst model II defines geometric variates taking the values 1, 2, 3, ... By their definition, it is clear that the population remains constant in size for all the models mentioned, and it is easy to verify that condition (3) is satisfied.

III. Genetic Behaviour of Populations

We come now to the effect of family structure on the genetic behaviour of a population, in particular after a large number of generations. The population is assumed to be diploid, and the genetic factor of interest is diallelic, so that the individuals are either of genotype *aa*, *Aa*, or *AA*. This is the formulation for autosomal genes, but we shall later deal with sex-linked genes, and then the male genotypes are either *a* or *A*. For the autosomal case, write k_t, u_t, l_t as the number of male individuals at the tth generation whose genotypes are *aa*, *Aa*, and *AA* respectively. Similarly, we define the numbers r_t, v_t, s_t for the females. If the sex numbers are N_1 and N_2, then the number of heterozygotes in either sex are $u_t = N_1-k_t-l_t$, $v_t = N_2-r_t-s_t$. The genetic state of the population at any time is determined by the four variates (k_t, l_t, r_t, s_t).

We consider a non-overlapping generation model for which all individuals are produced simultaneously at the death of the preceding generation and their genotypes are determined as if mating occurred between the sexes of that generation. For this model, the genetic state forms a Markov chain with points determined by the coordinates (k, l, r, s). There are two absorbings tates $(N_1, 0, N_2, 0)$ and $(0, N_1, 0, N_2)$

corresponding to homozygosity and gene fixation, and the transition probabilities from any state to any other state could be written down. By the general theory of Markov chains it can be shown that the probability that the system has not reached an absorbing state at time t ($t = 0, 1, 2, \ldots$) is asymptotically $C\lambda^t$, where t is measured in units of one generation, C is a constant depending on the initial state, and λ is the largest non-unit root of the matrix of transition probabilities. This matrix is too complicated to be written down explicitly, but by calculating the first and second moments of k, l, r, s at time $t+1$ in terms of those at time t it can be shown that λ is also the largest characteristic root governing these difference equations. We proceed by the latter method; the determination of the constant C is more difficult, and has been carried out only for the simpler haploid models. It will therefore not be attempted here.

First we take the case when permanent marriage does not occur, and assume random mating subject to the restriction that the generating functions for the number of offspring per individual are $P_1(z)$ and $P_2(z)$ for males and females respectively. At each generation the sex numbers will be constant at N_1 and N_2, so equations (3) must hold. We write x_{ij} for the numbers of offspring produced by all matings between individuals of specified genotypes according to the scheme:

		Males			
		aa	Aa	AA	
	aa	x_{11}	x_{12}	x_{13}	$x_{1.}$
Females	Aa	x_{21}	x_{22}	x_{23}	$x_{2.}$
	AA	x_{31}	x_{32}	x_{33}	$x_{3.}$
		$x_{.1}$	$x_{.2}$	$x_{.3}$	N

....(11)

Thus $x_{.j} = \sum_i x_{ij}$ and so on. If the parent generation is the tth, then we obtain from the offspring distributions, equation (3), that

$$\left.\begin{aligned} E(x_{.1}) &= k_t N N_1^{-1}, & E(x_{1.}) &= r_t N N_2^{-1}, \\ E(x_{.3}) &= l_t N N_1^{-1}, & E(x_{3.}) &= s_t N N_2^{-1}. \end{aligned}\right\} \quad \ldots\ldots\ldots\ldots(12)$$

Furthermore, using the values of c_1 and c_2 from equation (4) we get

$$\left.\begin{aligned} \operatorname{var}(x_{.1}) &= k_t\, \sigma_1^2\{1-(k_t-1)(N_1-1)^{-1}\}, \\ \operatorname{var}(x_{.3}) &= l_t\, \sigma_1^2\{1-(l_t-1)(N_1-1)^{-1}\}, \\ \operatorname{var}(x_{1.}) &= r_t\, \sigma_2^2\{1-(r_t-1)(N_2-1)^{-1}\}, \\ \operatorname{var}(x_{3.}) &= s_t\, \sigma_2^2\{1-(s_t-1)(N_2-1)^{-1}\}, \\ \operatorname{cov}(x_{.1}, x_{.3}) &= -k_t\, l_t\, \sigma_1^2(N_1-1)^{-1}, \\ \operatorname{cov}(x_{1.}, x_{3.}) &= -r_t\, s_t\, \sigma_2^2(N_2-1)^{-1}. \end{aligned}\right\} \quad \ldots(13)$$

Of these offspring exactly N_1 are to be male, N_2 female, but apart from these restrictions, we assume random sex distributions. If we write $x_{ij} = m_{ij}+f_{ij}$, where m_{ij} and f_{ij} are the numbers of male and female offspring from the mating type (i, j), we can express this condition by representing the m_{ij} and f_{ij} in a 2×9 contingency table as follows:

m_{11}	m_{12}	m_{13}	m_{21}	m_{22}	m_{23}	m_{31}	m_{32}	m_{33}	N_1
f_{11}	f_{12}	f_{13}	f_{21}	f_{22}	f_{23}	f_{31}	f_{32}	f_{33}	N_2
x_{11}	x_{12}	x_{13}	x_{21}	x_{22}	x_{23}	x_{31}	x_{32}	x_{33}	N

The x_{11} offspring from matings of type $(aa\times aa)$ are all of genotype aa, the x_{12} offspring from matings $(aa\times Aa)$ are a mixture of genotypes aa and Aa, each formed with probability $\frac{1}{2}$, and similar results hold for each of the other mating types. We write

$$\begin{aligned} m_{ij} &= k_{ij}+u_{ij}+l_{ij}, \\ f_{ij} &= r_{ij}+v_{ij}+s_{ij}, \end{aligned}$$

where k_{ij} is the number of male offspring of type aa from matings of type (i, j) and so on. If we write p_{ij}, q_{ij} for the probabilities of a single offspring from a mating type (i, j) being of genotype aa or AA, then the total genotypic outputs in males and females have the trinomial distributions

$$\left.\begin{aligned} Pr\{k_{ij}, u_{ij}, l_{ij}\} &= \frac{m_{ij}!}{k_{ij}!\, u_{ij}!\, l_{ij}!}\, p_{ij}^{k_{ij}}(1-p_{ij}-q_{ij})^{u_{ij}}\, q_{ij}^{l_{ij}}, \\ Pr\,\{r_{ij}, v_{ij}, s_{ij}\} &= \frac{f_{ij}!}{r_{ij}!\, v_{ij}!\, s_{ij}!}\, p_{ij}^{r_{ij}}(1-p_{ij}-q_{ij})^{v_{ij}}\, q_{ij}^{s_{ij}}, \end{aligned}\right\} \quad ..(\mathbf{14})$$

where p_{ij} and q_{ij} are given in the following table:

i, j	11	12	13	21	22	23	31	32	33
p_{ij}	1	$\frac{1}{2}$	0	$\frac{1}{2}$	$\frac{1}{4}$	0	0	0	0
q_{ij}	0	0	0	0	$\frac{1}{4}$	$\frac{1}{2}$	0	$\frac{1}{2}$	1

Finally the genotype numbers in the offspring generation are $k_{t+1} = \Sigma k_{ij}$, $l_{t+1} = \Sigma l_{ij}$, $r_{t+1} = \Sigma r_{ij}$, $s_{t+1} = \Sigma s_{ij}$. We now consider the first- and second-order moments:

$$\begin{aligned} p_{t+1} &= N_1^{-1}E(k_{t+1}+l_{t+1}), \\ q_{t+1} &= N_2^{-1}E(r_{t+1}+s_{t+1}), \\ v_{t+1} &= N_1^{-1}N_2^{-1}E(k_{t+1}-l_{t+1})(r_{t+1}-s_{t+1}), \\ a_{t+1} &= N_1^{-2}E(k_{t+1}-l_{t+1})^2, \\ b_{t+1} &= N_2^{-2}E(r_{t+1}-s_{t+1})^2. \end{aligned}$$

We have to express these quantities in terms of the same quantities at generation t.

First consider expectations conditional on the values of m_{ij} and f_{ij}. From the distributions (**14**) we have

$$
\begin{aligned}
E(k_{ij}) &= m_{ij}\, p_{ij}, & E(k_{ij}^2) &= m_{ij}\, p_{ij}+m_{ij}(m_{ij}-1)p_{ij}^2, \\
E(l_{ij}) &= m_{ij}\, q_{ij}, & E(l_{ij}^2) &= m_{ij}\, q_{ij}+m_{ij}(m_{ij}-1)q_{ij}^2, \\
E(k_{ij}\, l_{ij}) &= m_{ij}(m_{ij}-1)p_{ij}q_{ij}, & E(k_{ij}l_{lm}) &= m_{ij}\, m_{lm}\, p_{ij}\, p_{lm},
\end{aligned}
$$

and k_{ij} and k_{lm} are independent for $(i, j) \neq (l, m)$. Similar relations hold for the female variates. In this way we find

$$
\begin{aligned}
p_{t+1} &= N_1^{-1}E(m_{11}+\tfrac{1}{2}m_{12}+\tfrac{1}{2}m_{21}+\tfrac{1}{2}m_{22}+\tfrac{1}{2}m_{23}+\tfrac{1}{2}m_{32}+m_{33}), \\
&= \tfrac{1}{2}+\tfrac{1}{2}N_1^{-1}E(m_{11}+m_{33}-m_{13}-m_{31}),
\end{aligned}
$$

and similarly

$$
q_{t+1} = \tfrac{1}{2}+\tfrac{1}{2}N_2^{-1}E(f_{11}+f_{33}-f_{13}-f_{31}).
$$

Since the distributions of genotype frequencies in males and females are independent so long as the m_{ij} and f_{ij} are kept fixed we have

$$
\begin{aligned}
v_{t+1} = N_1^{-1}N_2^{-1}E(m_{11}&+\tfrac{1}{2}m_{12}+\tfrac{1}{2}m_{21}-\tfrac{1}{2}m_{23}-\tfrac{1}{2}m_{32}-m_{33}) \\
&\times(f_{11}+\tfrac{1}{2}f_{12}+\tfrac{1}{2}f_{21}-\tfrac{1}{2}f_{23}-\tfrac{1}{2}f_{32}-f_{33}),
\end{aligned}
$$

and

$$
\begin{aligned}
a_{t+1} &= N_1^{-2}E\{(m_{11}+\tfrac{1}{2}m_{12}+\tfrac{1}{2}m_{21}-\tfrac{1}{2}m_{23}-\tfrac{1}{2}m_{32}-m_{33})^2 \\
&\qquad +\tfrac{1}{4}m_{12}+\tfrac{1}{4}m_{21}+\tfrac{1}{2}m_{22}+\tfrac{1}{4}m_{23}+\tfrac{1}{4}m_{32}\}, \\
b_{t+1} &= N_2^{-2}E\{(f_{11}+\tfrac{1}{2}f_{12}+\tfrac{1}{2}f_{21}-\tfrac{1}{2}f_{23}-\tfrac{1}{2}f_{32}-f_{33})^2 \\
&\qquad +\tfrac{1}{4}f_{12}+\tfrac{1}{4}f_{21}+\tfrac{1}{2}f_{22}+\tfrac{1}{4}f_{23}+\tfrac{1}{4}f_{32}\}.
\end{aligned}
$$

We now have to calculate these expectations conditional on the values of the x_{ij}. Using standard results for the conditional expectations of the entries in a contingency table (e.g. see Wilks 1946, p. 216) we have

$$
\begin{aligned}
E(m_{ij}) &= x_{ij}N_1N^{-1}, \\
E(f_{ij}) &= x_{ij}N_2N^{-1}, \\
E(m_{ij}^2) &= x_{ij}(x_{ij}-1)N_1(N_1-1)N^{-1}(N-1)^{-1}+x_{ij}N_1N^{-1}, \\
E(f_{ij}^2) &= x_{ij}(x_{ij}-1)N_2(N_2-1)N^{-1}(N-1)^{-1}+x_{ij}N_2N^{-1}, \\
E(m_{ij}\, f_{ij}) &= x_{ij}\,(x_{ij}-1)N_1N_2N^{-1}(N-1)^{-1},
\end{aligned}
$$

and for $(i, j) \neq (l, m)$

$$
\begin{aligned}
E(m_{ij}\, m_{lm}) &= x_{ij}\, x_{lm}N_1(N_1-1)N^{-1}(N-1)^{-1}, \\
E(f_{ij}\, f_{lm}) &= x_{ij}\, x_{lm}N_2(N_2-1)N^{-1}(N-1)^{-1}, \\
E(m_{ij}\, f_{lm}) &= x_{ij}\, x_{lm}N_1N_2N^{-1}(N-1)^{-1}.
\end{aligned}
$$

Using these results and also the fact that

$$x_{11}+\tfrac{1}{2}x_{12}+\tfrac{1}{2}x_{21}-\tfrac{1}{2}x_{23}-\tfrac{1}{2}x_{32}-x_{33} = \tfrac{1}{2}(x_{1.}-x_{3.}+x_{.1}-x_{.3}),$$

we find

$$\left.\begin{aligned} p_{t+1} &= q_{t+1} = \tfrac{1}{2}+\tfrac{1}{2}N^{-1}E(x_{11}+x_{33}-x_{13}-x_{31}),\\ v_{t+1} &= N^{-1}(N-1)^{-1}E\{(x_{11}+\tfrac{1}{2}x_{12}+\tfrac{1}{2}x_{21}-\tfrac{1}{2}x_{23}-\tfrac{1}{2}x_{32}-x_{33})^2\\ &\qquad -(x_{11}+\tfrac{1}{4}x_{12}+\tfrac{1}{4}x_{21}+\tfrac{1}{4}x_{23}+\tfrac{1}{4}x_{32}+x_{33})\},\\ &= N^{-1}(N-1)^{-1}\,E\{\tfrac{1}{4}(x_{1.}-x_{3.}+x_{.1}-x_{.3})^2-\tfrac{1}{4}(x_{1.}+x_{3.}+x_{.1}+x_{.3})\\ &\qquad -\tfrac{1}{2}(x_{11}+x_{33}-x_{13}-x_{31})\},\\ a_{t+1} &= (1-N_1^{-1})v_{t+1}+N_1^{-1}p_{t+1},\\ b_{t+1} &= (1-N_2^{-1})v_{t+1}+N_2^{-1}q_{t+1}. \end{aligned}\right\} \quad ..(\mathbf{15})$$

The last two equations are obtained by comparing the values of a_{t+1} and b_{t+1} expressed as expectations conditional on fixed values of x_{ij} with those obtained for the other three quantities. Now regarding the x_{ij} as variates of a contingency table (**11**) with fixed row and column totals, we have the conditional expectations $E(x_{ij}) = x_{i.}x_{.j}N^{-1}$, and then taking expectations according to (**12**), (**13**), we get

$$E(x_{11}+x_{33}-x_{13}-x_{31}) = E(k_t-l_t)(r_t-s_t)NN_1^{-1}N_2^{-1},$$

$$\begin{aligned} &E\{(x_{1.}-x_{3.}+x_{.1}-x_{.3})^2-(x_{1.}+x_{3.}+x_{.1}+x_{.3})\}\\ &\quad = E(k_t-l_t)^2(N^2N_1^{-2}-\sigma_1^2(N_1-1)^{-1})+E(r_t-s_t)^2(N^2N_2^{-2}-\sigma_2^2(N_2-1)^{-1})\\ &\qquad +2E(k_t-l_t)(r_t-s_t)N^2N_1^{-1}N_2^{-1}-E(k_t+l_t)(NN_1^{-1}-\sigma_1^2N_1(N_1-1)^{-1})\\ &\qquad -E(r_t+s_t)(NN_2^{-1}-\sigma_2^2N_2(N_2-1)^{-1}). \end{aligned}$$

Thus our difference equations may be written

$$\left.\begin{aligned} p_{t+1} &= q_{t+1} = \tfrac{1}{2}+\tfrac{1}{2}v_t,\\ v_{t+1} &= N^{-1}(N-1)^{-1}\{\tfrac{1}{4}a_t(N^2-N_1^2\sigma_1^2(N_1-1)^{-1})+\tfrac{1}{4}b_t(N^2-N_2^2\sigma_2^2(N_2-1)^{-1})\\ &\qquad -\tfrac{1}{4}p_t(N-N_1^2\sigma_1^2(N_1-1)^{-1})-\tfrac{1}{4}q_t(N-N_2^2\sigma_2^2(N_2-1)^{-1})\\ &\qquad +\tfrac{1}{2}N(N-1)v_t\},\\ a_{t+1} &= (1-N_1^{-1})v_{t+1}+N_1^{-1}p_{t+1},\\ b_{t+1} &= (1-N_2^{-1})v_{t+1}+N_2^{-1}q_{t+1}. \end{aligned}\right\}$$

$$\ldots\ldots\ldots\ldots(\mathbf{16})$$

Substituting for q_t, a_t, and b_t in the right-hand side of v_{t+1} we get finally two difference equations for the quantities p and v, the other moments being simple functions of these.

$$\begin{aligned} p_{t+1} &= \tfrac{1}{2}+\tfrac{1}{2}v_t,\\ v_{t+1} &= \tfrac{1}{4}N^{-1}(N-1)^{-1}\{(N^3N_1^{-1}N_2^{-1}-2N+N_1\sigma_1^2+N_2\sigma_2^2)p_t\\ &\qquad +(4N^2-2N-N^3N_1^{-1}N_2^{-1}-N_1\sigma_1^2-N_2\sigma_2^2)v_t\}. \end{aligned}$$

The solution of these equations has the form

$$\begin{aligned} p_t &= 1-\alpha_1\lambda_1^t-\alpha_2\lambda_2^t, \\ v_t &= 1-\beta_1\lambda_1^t-\beta_2\lambda_2^t, \end{aligned}$$

where the λ's are the characteristic roots of the matrix of coefficients in the equations, and the α's and β's are constants chosen to satisfy the initial conditions. The characteristic equation is

$$\begin{aligned} &\lambda^2-\lambda\tfrac{1}{4}N^{-1}(N-1)^{-1}(4N^2-2N-N^3N_1^{-1}N_2^{-1}-N_1\sigma_1^2-N_2\sigma_2^2)-\tfrac{1}{8}N^{-1}(N-1)^{-1} \\ &\qquad \times(N^3N_1^{-1}N_2^{-1}-2N+N_1\sigma_1^2+N_2\sigma_2^2) = 0, \end{aligned}$$

and the larger root, which governs the rate of approach to homozygosity, is approximately

$$\left.\begin{aligned} \lambda &\doteqdot 1-\tfrac{1}{8}N^{-2}(N^3N_1^{-1}N_2^{-1}-2N+N_1\sigma_1^2+N_2\sigma_2^2), \\ &= 1-\tfrac{1}{8}N^{-2}(N_1P_1''(1)+N_2P_2''(1)). \end{aligned}\right\} \quad \ldots\textbf{(17)}$$

Thus we have related the rate of progress to homozygosity to the offspring distributions whose generating functions are $P_1(z)$ and $P_2(z)$.

By considering the expectations of k_{t+1}, l_{t+1}, r_{t+1}, and s_{t+1} in terms of those at generation t, we can easily find the probabilities of ultimate absorption in the two absorbing states $(N_1, 0, N_2, 0)$ and $(0, N_1, 0, N_2)$. Thus the probability of absorption in the former is the ultimate value of $N_1^{-1}E(k_{t+1})$ which may be denoted $N_1^{-1}E(k_\infty)$. We know already that $N_1^{-1}E(k_\infty+l_\infty) = 1$. It may be shown, by successively taking conditional expectations as before, that

$$E\{N_1^{-1}(k_\infty-l_\infty)+N_2^{-1}(r_\infty-s_\infty)\} = N_1^{-1}(k_0-l_0)+N_2^{-1}(r_0-s_0),$$

where the subscript zero denotes the initial value, so we have

$$N_1^{-1}E(k_\infty-l_\infty) = \tfrac{1}{2}N_1^{-1}(k_0-l_0)+\tfrac{1}{2}N_2^{-1}(r_0-s_0).$$

Therefore the probability of fixation of the a gene is

$$N_1^{-1}E(k_\infty) = \tfrac{1}{2}+\tfrac{1}{4}N_1^{-1}(k_0-l_0)+\tfrac{1}{4}N_2^{-1}(r_0-s_0),$$

which is the mean of the relative frequencies of the a gene in the males and females of the initial population. We notice in particular that it is only equal to the overall initial frequency of a when $N_1 = N_2$.

In the above model each offspring is the result of an independent random mating between the adults of the previous generation, subject to the restriction imposed by the probability distribution of the numbers of offspring. Consider now a model in which "marriage" occurs, i.e. permanent matings between pairs of the opposite sex. We now take $N_1 = N_2 = \frac{1}{2}N$ so that each individual has one mate. We must then have $\sigma_1^2 = \sigma_2^2$ $(= \sigma^2$ say) since the male and female parents of a family must have the same number of offspring. To construct such a model we proceed as before but introduce an additional set of variates. We suppose that y_{ij} are the numbers of permanent matings between parents of genotypes (i, j) in accordance with the following scheme:

		Males			
		aa	Aa	AA	
Females	aa	y_{11}	y_{12}	y_{13}	r_t
	Aa	y_{21}	y_{22}	y_{23}	v_t
	AA	y_{31}	y_{32}	y_{33}	s_t
		k_t	u_t	l_t	$\frac{1}{2}N$

The y_{ij} families have x_{ij} offspring in accordance with the probability distribution of the numbers of offspring and the correlation between numbers of offspring of different parents already calculated. Thus the previous equations giving expectations in terms of the x_{ij} remain valid and only the relationships between the x_{ij} and k_t, l_t, r_t, s_t are modified. Using (**3**) and (**4**) we have

$$\begin{aligned}
E(x_{ij}) &= 2y_{ij},\\
E(x_{ij}^2) &= y_{ij}\sigma^2\{1-(y_{ij}-1)(\tfrac{1}{2}N-1)^{-1}\}+4y_{ij}^2,\\
E(x_{ij}\,x_{lm}) &= -\sigma^2 y_{ij}\,y_{lm}(\tfrac{1}{2}N-1)^{-1}+4y_{ij}\,y_{lm}, \qquad (i,j)\neq(l,m)
\end{aligned}$$

and furthermore

$$\begin{aligned}
E(y_{11}) &= 2N^{-1}k_t r_t, \qquad E(y_{33}) = 2N^{-1}l_t s_t\\
E(y_{13}) &= 2N^{-1}l_t r_t, \qquad E(y_{31}) = 2N^{-1}k_t s_t\\
y_{11}+\tfrac{1}{2}y_{12}+\tfrac{1}{2}y_{21}-\tfrac{1}{2}y_{23}-\tfrac{1}{2}y_{32}-y_{33} &= \tfrac{1}{2}(k_t-l_t+r_t-s_t).
\end{aligned}$$

Inserting these in equations (**15**) we obtain

$$\begin{aligned}
p_{t+1} = q_{t+1} &= \tfrac{1}{2}+2N^{-2}E(k_t-l_t)(r_t-s_t),\\
v_{t+1} &= N^{-1}(N-1)^{-1}\{E[(k_t-l_t)^2+(r_t-s_t)^2][1-\tfrac{1}{4}\sigma^2(\tfrac{1}{2}N-1)^{-1}]\\
&\qquad +\tfrac{1}{2}E(k_t+l_t+r_t+s_t)[\tfrac{1}{4}N\sigma^2(\tfrac{1}{2}N-1)^{-1}-1]\\
&\qquad +2N^{-1}(N-1)E(k_t-l_t)(r_t-s_t)\},\\
a_{t+1} &= (1-2N^{-1})v_{t+1}+2N^{-1}p_{t+1},\\
b_{t+1} &= (1-2N^{-1})v_{t+1}+2N^{-1}q_{t+1},
\end{aligned}$$

which are identical with those of (**16**) when N_1, N_2 are replaced by $\frac{1}{2}N$, and σ_1^2 and σ_2^2 by σ^2. Thus the rate of progress to homozygosity is unaffected by the occurrence of permanent marriage. It is also true that the probabilities of ultimate fixation of one or other gene are unaffected by marriage.

Without giving any details, we shall state the results obtained by similar analyses for sex-linked genes. If the male sex is heterogametic, the female homogametic, their respective genotypes are a or A; aa, Aa, or AA. Suppose that the numbers of individuals having these genotypes at the tth generation are k_t, N_1-k_t, and r_t, $N_2-r_t-s_t$, s_t, then the state of the population is determined by the three variates (k_t, r_t, s_t) and the two possible absorbing states are $(N_1, N_2, 0)$ and $(0, 0, N_2)$. It can be shown that the rate of approach to one or other of these states is

$$\lambda \doteqdot 1-\tfrac{1}{9}N^{-2}(N_1P_1''(1)+2N_2P_2''(1)), \qquad \ldots\ldots\ldots\ldots(18)$$

where the symbols have the same meanings as before. The probability of ultimate fixation of the a genes is now

$$\tfrac{1}{2}+\tfrac{1}{6}(2k_0-N_1)N_1^{-1}+\tfrac{1}{3}(r_0-s_0)N_2^{-1}.$$

We may verify that the rates found here substantiate the equivalence between two populations of different size and offspring distribution considered in the previous section. For a population of size N and generating functions (5), the rates are

$$\lambda \doteqdot 1-\tfrac{1}{8}N^{-1}\pi^2R''(1)$$

for autosomal genes, and

$$\lambda \doteqdot 1-\tfrac{1}{6}N^{-1}\pi^2R''(1)$$

for sex-linked genes. Alternatively, for the population of size $\tfrac{1}{2}NR'(1)$ and generating functions (6), we get

$$\lambda \doteqdot 1-\tfrac{1}{4}N^{-1}[R'(1)]^{-1}\pi R''(1)$$

for autosomal genes, and

$$\lambda \doteqdot 1-\tfrac{1}{3}N^{-1}[R'(1)]^{-1}\pi R''(1)$$

for sex-linked genes. Because these pairs of rates may apply to the same population under two methods of enumeration, they must be identical. For this to be true, we need $R'(1) = 2\pi^{-1}$, which is just the condition required for a population to have constant size.

The above diploid models assume non-overlapping generations. As particular cases, consider first Wright's model having generating functions (7). For these, the second derivatives are

$$P_1''(1) = N(N-1)N_1^{-2},$$
$$P_2''(1) = N(N-1)N_2^{-2},$$

and so the rate of approach to homozygosity is approximately

$$\lambda \doteqdot 1-N/8N_1N_2$$

for autosomal genes, and

$$\lambda \doteqdot 1-(N_2+2N_1)/9N_1N_2$$

for sex-linked genes. These two results had been found previously by Wright (1931, 1933) by a different method.

For a model in which all males have exactly NN_1^{-1} offspring, all females exactly NN_2^{-1}, the second derivatives are, from (8),

$$P_1''(1) = NN_1^{-1}(NN_1^{-1}-1),$$

$$P_2''(1) = NN_2^{-1}(NN_2^{-1}-1),$$

so that the rate of approach to homozygosity is

$$\lambda \doteqdot 1-\tfrac{1}{8}\mathrm{N}^{-1}(N^2N_1^{-1}N_2^{-1}-2),$$
$$\lambda \doteqdot 1-\tfrac{1}{9}N^{-1}\{N(N_2+2N_1)N_1^{-1}N_2^{-1}-3\},$$

for autosomal and sex-linked genes, respectively.

In the preceding section, two other models were considered, and their generating functions obtained. Although both models were for populations with overlapping generations, it is interesting to see the effect this has by comparing the results obtained in previous papers for these models with those derived here for non-overlapping generations. Moran (1958) found for his overlapping generation model that the rate of approach to homozygosity for autosomal genes was

$$\lambda \doteqdot 1-N/4N_1N_2.$$

Compare this with the result for a population having the same generating functions (**9**) but having non-overlapping generations. From (**17**) we get

$$\begin{aligned}\lambda &\doteqdot 1-\tfrac{1}{8}N^{-2}(N_1 2N(N-1)N_1^{-2}+N_2 2N(N-1)N_2^{-2}),\\ &\doteqdot 1-N/4N_1N_2,\end{aligned}$$

which is identical with the previous expression. Thus it would seem that a population with overlapping generations would have asymptotically the same rate of progress to homozygosity as one with non-overlapping generations and the same offspring distributions. That this, however, cannot be true in general is shown by the theory of the other overlapping generation model II. In this case, Watterson (unpublished data) found the rate of approach to homozygosity for autosomal genes as

$$\lambda \doteqdot 1-N/8N_1N_2$$

for random mating, whereas for a non-overlapping population with the same generating functions (**10**), the present theory gives the rate as

$$\lambda \doteqdot 1-(N_1^2+N_2^2)/4NN_1N_2.$$

This root is not identical with the previous one except when $N_1 = N_2$. From this we see that (**17**) cannot always be extended to overlapping generation models and the determination of the conditions under which this is possible must await the development of a general theory to cover the latter. It appears that this will be a difficult task, for the state of such a general population needs the specification of all individuals ages as well as their genotypes, and such random processes present theoretical difficulties not yet overcome.

IV. REFERENCES

FISHER, R. A. (1939).—Stage of development as a factor influencing the variance in the number of offspring, frequency of mutants and related quantities. *Ann. Eugen., Lond.* **9**: 406–8.

HALDANE, J. B. S. (1939).—The equilibrium between mutation and random extinction. *Ann. Eugen., Lond.* **9**: 400–5.

LI, C. C. (1955)—"Population Genetics." (University of Chicago Press.)

MORAN, P. A. P. (1958).—Random processes in genetics. *Proc. Camb. Phil. Soc.* **54**: 60–71.

WILKS, S. S. (1946).—"Mathematical Statistics." (Princeton University Press.)

WRIGHT, S. (1931).—Evolution in Mendelian populations. *Genetics* **16**: 97–159.

WRIGHT, S. (1933).—Inbreeding and homozygosis. *Proc. Nat. Acad. Sci., Wash.* **19**: 411–19.

UPTAKE AND TRANSLOCATION OF CHLORINE IN CITRUS CUTTINGS DURING AND AFTER A SHORT SALT TREATMENT

By H. Groenewegen,* D. Bouma,* and C. T. Gates*

[*Manuscript received July* 15, 1958]

Summary

Rooted citrus cuttings were subjected to a short salinity stress and after salt removal the chlorine redistribution was studied over a period of 82 days.

No significant change was found in the chlorine content of leaves present at the date of salt removal.

No significant loss of chlorine from the plant as a whole occurred. Chlorine moved upwards; roots and old-cycle stems showed an initial rapid decrease in chlorine, after which the release of chlorine previously accumulated in these parts was slow.

I. Introduction

Redistribution of mineral elements in the plant has recently been reviewed by Williams (1955) and Gauch (1957). In the case of nitrogen, phosphorus, and potassium the translocation occurs mainly from older to younger plant parts and appears therefore to be largely dependent on a metabolic gradient. No evidence for a considerable translocation of calcium from old leaves to younger plant parts has been found. For other elements such as zinc and copper the translocation appears to be dependent on experimental conditions.

Little evidence is available on the redistribution of chlorine. Ulrich and Ohki (1956) found no movement of chlorine from old to young leaves of chlorine-deficient sugar-beets. Phillis and Mason (1942) found a moderate diurnal fluctuation in chlorine contents of cotton leaves, an increase occurring during the day and a decrease during the night. Harding, Miller, and Fireman (1957) found much higher chlorine contents in the lower leaves of sprinkler-irrigated citrus trees than in the upper leaves; the latter were not normally covered by the sprays. This indicated that chlorine absorbed from the irrigation water by the lower leaves was not readily translocated to the upper leaves.

It is well known that the concentration of salt in the soil solution fluctuates greatly between irrigations and different seasons. It would therefore be of interest to establish whether salts accumulated in plant parts during a salinity stress are readily translocated to younger plant parts. This becomes particularly important in the case of citrus where leaves remain on the tree for periods of up to three years. In addition, citrus is considered to be sensitive to even low chlorine contents in its tissues (Chapman 1949). A ready translocation to developing plant parts would in this case be more serious than in crops less sensitive to chlorine.

This paper reports an experiment with citrus cuttings in which the redistribution of chlorine was studied after a short salt treatment.

---

*Irrigation Research Station, C.S.I.R.O., Griffith, N.S.W.

II. METHODS

Rooted cuttings of Washington navel oranges with 2–4 leaves were transplanted into quartz sand. The composition of the basal nutrient solution in m-equiv/l was: nitrate 10, sulphate 3, phosphate 1, chloride 0·15, calcium 7, magnesium 3, potassium 3, and sodium 0·3. The pH was kept between 6 and 7. This solution was leached through the pots three times daily in 100-ml lots.

For the salt treatment a mixture of calcium, magnesium, potassium, and sodium chlorides was added to the basal nutrient solution and the cations occurred in the same ratios as in the basal nutrient solution. At full strength the salt composition in m-equiv/l was: chloride 200, calcium 105, potassium 45, magnesium 45, and sodium 4·5.

Salt treatment was begun 74 days after transplanting of the cuttings. The salt concentration was increased in steps of 25 m-equiv/l per day. Each can was flushed with 1 l. of solution, an amount approximately five times the field capacity of the sand used in the cultures. The final strength of 200 m-equiv/l was maintained for 3 days. At the end of the salt treatment the salts were removed, first by leaching with rainwater, and then with basal nutrient solution till the effluent had the same chloride content as the basal nutrient solution.

Four plants from both control and treated series were harvested on each of four occasions. These took place 0, 5, 25, and 82 days after leaching the salt from the treated plants (May 20, 1955). These harvests will be referred to as H_1, H_2, H_3, and H_4 respectively.

The plants were separated into different cycles of growth. The parts of the original cutting will be referred to as old cycle, and the subsequent growth as first and second cycle. Laminae, petioles, and stems of each cycle were harvested separately. Roots were rinsed in distilled water. All plant parts were dried rapidly at 80°C.

Chlorine was determined according to Best (1950).

III. RESULTS

(a) *Dry Weight*

The dry weight of the different plant parts of control and salt-treated plants is shown in Table 1. Dry weight differences between treatments were rather small, and not significant. A slow increase in dry weight of the whole plant between H_1 and H_3 was contributed by increases in roots, stems, and early growth of second-cycle laminae.

The second cycle began to develop before H_3, and grew rapidly between H_3 and H_4. This resulted in a large dry weight increase over this period, with the second-cycle parts contributing approximately 80 per cent. to this increase. Concurrent with this rapid development of the second cycle, the old- and first-cycle stems and the roots showed a much greater increase in dry weight than in the preceding period. Old- and first-cycle laminae and petioles did not show any appreciable increase in dry weight between H_3 and H_4.

(*b*) *Chlorine Uptake and Translocation*

The chlorine percentages of the different plant parts at the four harvest dates are shown in Figure 1. At salt removal (H_1) the chlorine percentage in the salt-treated plants was highest in the roots and decreased in the order: stem, petioles, and laminae. After salt removal (H_1–H_4) no marked change was found in the old- and first-cycle laminae of these plants. Some decrease took place in the old- and first-cycle petioles, while large decreases occurred in the stems and roots. The percentage of chlorine in all second-cycle parts decreased during the rapid growth between H_3 and H_4.

TABLE 1

DRY WEIGHT (G) OF WHOLE PLANT AND PRINCIPAL PARTS OF ROOTED ORANGE CUTTINGS

Plant Part	Cycle	Treatment	May 20 (H_1)	May 25 (H_2)	June 14 (H_3)	August 10 (H_4)
Laminae	Old	Control	1·275	1·427	1·482	1·303
		Salt	1·373	1·305	1·135	1·476
	First	Control	1·865	2·159	2·153	2·468
		Salt	2·002	1·914	1·728	2·479
	Second	Control	—	—	0·277	9·160*
		Salt	—	0·003	0·531	8·844
Petioles	Old	Control	0·052	0·052	0·055	0·061
		Salt	0·052	0·047	0·047	0·057
	First	Control	0·098	0·128	0·125	0·147
		Salt	0·115	0·108	0·104	0·118
	Second	Control	—	—	0·036	0·476
		Salt	—	—	0·053	0·439
Stems	Old	Control	0·356	0·484	0·581	1·291
		Salt	0·412	0·442	0·535	1·303
	First	Control	0·456	0·607	0·656	1·456
		Salt	0·515	0·527	0·571	1·105
	Second	Control	—	—	0·125	1·602†
		Salt	—	—	0·129	1·321
Roots		Control	0·836	1·209	1·412	2·674
		Salt	0·920	1·151	1·208	2·352
Whole plant		Control	4·937	6·065	6·901	20·637
Whole plant		Salt	5·389	5·497	6·039	19·494

*This value includes 0·090 g of third-cycle growth.

†This value includes 0·072 g of third-cycle growth.

Chlorine contents of the controls were much lower than those of the salt treatment, and as in the salt treatment decreases occurred in the old- and first-cycle stems, and in the second-cycle parts. The decrease of the chlorine percentage in the other plant parts of the control between H_3 and H_4 was of doubtful significance, because of experimental variability.

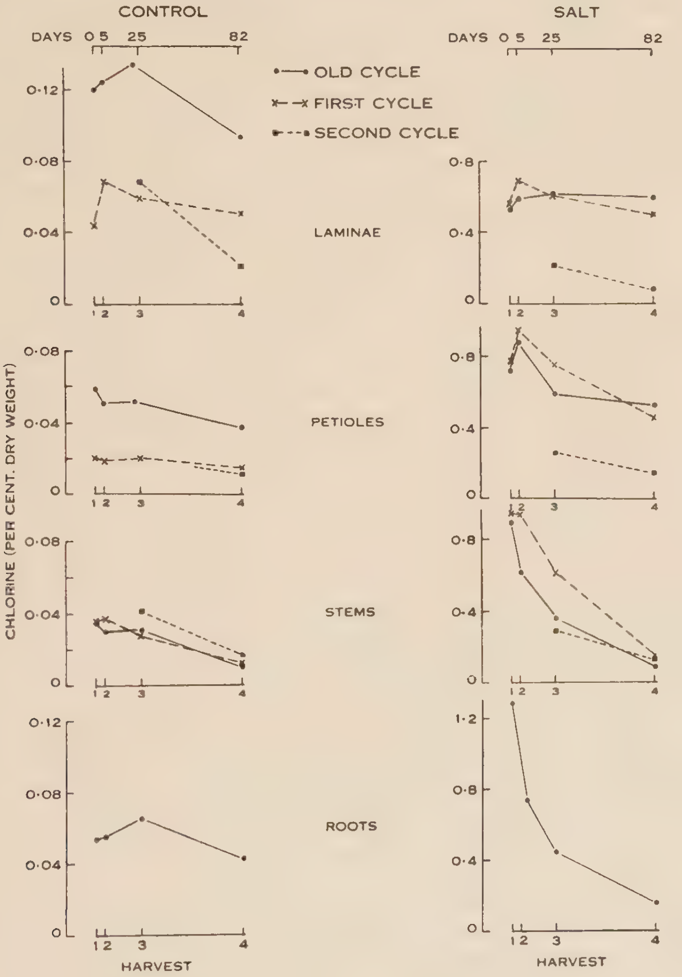


Fig. 1.—Per cent. of chlorine in the plant parts of rooted orange cuttings at different harvest dates after salt removal. (The scale for the control treatment is × 10 that for the salt treatment. The sample of second-cycle petioles of the control (H_3) was too small for analysis.)

Dilution effects in different plant parts, due to varying degrees of dry weight increases after salt removal, have occurred. For a study of the chlorine redistribution absolute amounts are therefore more relevant. These results are presented in

Figure 2. To calculate significant differences the data were transformed on a logarithmic basis, and these are presented in Table 2.

Chlorine in the roots of salt-treated plants decreased greatly, while that of the control plants increased with time. Eighty-two days after salt removal (H_4) the roots of salt-treated plants still contained greater amounts of chlorine than those of the control. In the salt-treated plants the old- and first-cycle stems showed a similar picture, but no appreciable change with time was found in the controls. Chlorine in the old- and first-cycle petioles of the salt-treated plants decreased somewhat with time, but this became significant only in the first-cycle petioles. No important changes were found in the old- and first-cycle petioles of the control plants.

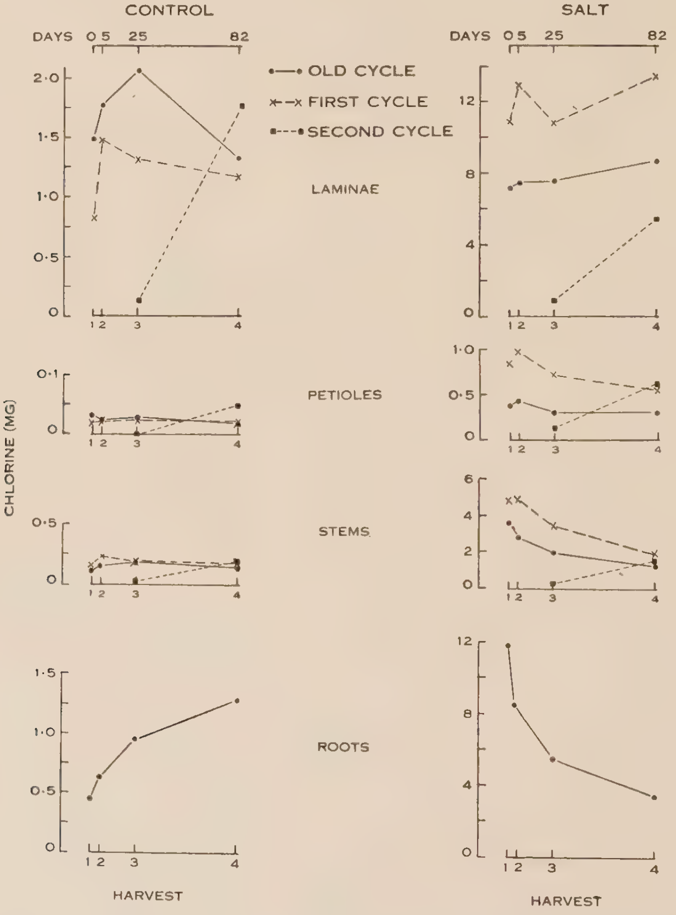


Fig. 2.—Absolute amounts of chlorine in the plant parts of rooted orange cuttings at different harvest dates after salt removal. (The scale for the control treatment is larger than for the salt treatment, and that for the petioles of both treatments is larger than that for the other plant parts.)

In contrast to the other plant parts existing at the date of salt removal, chlorine in the old- and first-cycle laminae tended to increase with time. In the controls trends are not consistent, and the decrease between H_3 and H_4 was not significant. In both control and salt-treated plants an increase of chlorine occurred in the second-cycle parts which developed approximately 3 weeks after salt removal. At H_4 these parts contained more chlorine in the salt treatment than in the control.

TABLE 2

LOG$_{10}$ CHLORINE CONTENT (MG × 100) OF PLANT PARTS IN ROOTED ORANGE CUTTINGS

Plant Part	Cycle	Treatment	May 20 (H_1)	May 25 (H_2)	June 14 (H_3)	August 10 (H_4)	Least Significant Difference ($P = 0·05$) for All Harvest Dates
Laminae	Old	Control	2·18	2·25	2·27	2·05	0·33
		Salt	2·85	2·86	2·88	2·94	
	First	Control	1·91	2·15	2·06	2·05	0·27
		Salt	3·03	3·11	3·02	3·07	
	Second	Control	—	—	0·71	2·23	0·13
		Salt	—	—	1·75	2·73	
Petioles	Old	Control	0·47	0·39	0·44	0·28	0·43
		Salt	1·54	1·57	1·47	1·48	
	First	Control	0·29	0·35	0·37	0·29	0·123
		Salt	1·93	1·99	1·86	1·73	
	Second	Control	—	—	—	0·68	0·59*
		Salt	—	—	1·04	1·75	
Stems	Old	Control	0·93	0·95	1·08	0·86	0·36
		Salt	2·56	2·42	2·28	2·05	
	First	Control	1·20	1·32	1·25	1·23	0·23
		Salt	2·66	2·68	2·52	2·20	
	Second	Control	—	—	0·52	1·28	0·54*
		Salt	—	—	1·49	2·17	
Roots		Control	1·65	1·75	1·97	1·99	0·32
		Salt	3·05	2·93	2·72	2·53	

*Least significant difference only applies for August 10.

To compare chlorine distribution between treatments and at different harvests the actual amounts of chlorine (mg) were plotted additively in Figure 3. In the controls the total amount of chlorine in the whole plant increased gradually with time, and this is mainly due to increases in second-cycle parts and in roots. Decrease of chlorine in roots and old stems of the salt-treated plants was rapid immediately after salt removal, but slowed down thereafter, e.g. logarithmic comparisons of absolute root data in Table 2 show an initial significant decrease in chlorine during 25 days (H_1–H_3) followed by a smaller insignificant decrease during 57 days (H_3–H_4). The losses of chlorine from roots and stems were at least partially compensated by increases in the laminae.

The total amount of chlorine in the whole plant of the salt treatments did not change significantly with time. The rather low value for total chlorine of H_3 was

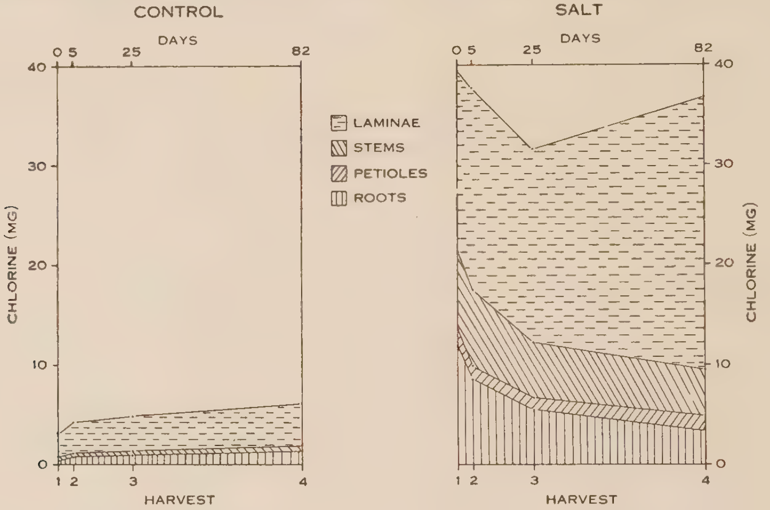


Fig. 3.—Total amount of chlorine in the whole plant, and its distribution in its plant parts of rooted orange cuttings at different harvest dates after salt removal. (The total amount of chlorine in stems and petioles of control plants is plotted as "chlorine stems".)

probably caused by the chance allocating of smaller plants to this harvest class. Chlorine uptake was shown to be correlated with plant size, and an indication of the initial plant sizes of the different harvests can be obtained from the following mean leaf area measurements taken at the date of treatment allocation: H_1, 207; H_2, 202; H_3, 180; H_4, 221 sq. cm.

IV. Discussion

The main finding of the reported experiment is the absence of any appreciable movement of chlorine from the older leaves of salt-treated plants to other plant parts. It should be borne in mind that the chlorine percentages in the leaves of this experiment were only moderately high, and much lower than those found by other workers (Cooper, Gorton, and Olson 1952). At such high levels different results might have been obtained. The results presented are in agreement with those of Ulrich and Ohki (1956) who found no appreciable movement of chlorine from older to younger leaves of chlorine-deficient sugar-beet plants.

The absence in this experiment of any appreciable movement from the older leaves would lead to higher chlorine concentrations in these leaves than in newly developing leaves. This could be an explanation for the premature leaf abscission of salt-affected citrus trees in the field, which may, nevertheless, be capable of developing a new growth cycle.

These observations are not necessarily in contradiction with the findings of Woolley, Broyer, and Johnston (1958) who observed a retranslocation of radioactive chlorine in tomatoes. They found during a 24-day period a loss of 30–50 per cent. of the chlorine previously absorbed in old leaves. This chlorine was retranslocated to other plant parts. In chlorine-deficient tomatoes the loss from the old leaves was relatively greater than from plants which were supplied with ample chlorine. Such a retranslocation may have occurred even in the salt-treated plants of the present experiment. If so, any outward movement of chlorine from old- and first-cycle laminae was either relatively small, or was compensated by a movement into the same organs.

The decrease in chlorine in the older stems and roots, the concurrent increase in the second-cycle parts, and the absence of any appreciable movement from the old- and first-cycle laminae indicates a predominantly upward movement. This finding is in accordance with Long (1943), who concluded from an experiment with approach-grafted tomatoes that chlorine moved only in the direction of the transpiration stream. Of course, in the present experiment, the possibility of a relatively small chlorine loss to the substrate cannot be excluded.

The rate of chlorine release from roots and old stems has been observed to fall with time after salt removal. This should be considered in relation to the higher chlorine content of these parts, relative to control, even 82 days following salt removal. The absolute amounts of chlorine at H_4 were $3 \cdot 5$ and $1 \cdot 2$ mg for roots and old stems in the salt treatment, and $1 \cdot 3$ and $0 \cdot 14$ mg respectively in the controls.

These observations find their most likely explanation in terms of ion movement in free space and ion accumulation into the vacuoles. Epstein (1956) and Briggs and Roberston (1957) suggested that there is a fairly free movement of ions within cell walls and possibly in some of the cytoplasm. From this "free space" individual cells would accumulate ions into their vacuoles. Several workers (Wiersum 1947; Helder 1952; Epstein 1956) have further found that ions, absorbed actively by the root cells, are not free to move to the shoots. The loss of chlorine in the present experiment may therefore have been mainly a movement from the free space of roots and old stems, followed by a much slower release from the vacuoles of these tissues. The data presented here do not allow of precise estimates of the nature of such movements nor was this the purpose of the investigations, but consideration of the data does suggest them as causal mechanisms.

This suggestion is enhanced by the results of Hylmö (1953), who used 3-week-old pea plants, and transferred them to distilled water after treatment with a calcium chloride solution for 22 hr. He found that only negligible leakage of calcium ions from the roots to the medium had occurred, while a considerable quantity of calcium was translocated from the roots to the shoots. Hylmö also suggests that part of the calcium lost from the roots after distilled water treatment for 22 hr was derived from the vacuoles of the roots.

In the citrus experiment upward movement of chlorine may, of course, have been further reduced by the considerable growth of stems and roots between H_3 and H_4. The formation of these new tissues will mitigate chlorine loss, by accumulating chlorine released from the vacuoles of cells formed earlier.

Steward, Prevot, and Harrison (1942) found that small amounts of bromide previously absorbed by barley roots, were entirely transported to the shoots after 4 days of growth in a bromide-free nutrient solution. Groenewegen (unpublished data) also found that barley roots of plants grown for a short time under sodium chloride treatment had lost 75 per cent. of their chlorine 5 days after the plants had been transferred to a basal nutrient solution. It has been shown that the rate of chlorine movement from the roots to the shoots of the citrus cuttings was very much slower.

V. Acknowledgments

The authors are greatly indebted to Dr. R. F. Williams, Division of Plant Industry, C.S.I.R.O., for his assistance with the preparation of the manuscript, and to Mr. G. A. McIntyre, Division of Mathematical Statistics, C.S.I.R.O., for the statistical analysis of the data.

They also wish to thank Mrs. E. M. Belford, Miss S. J. Byford, Mrs. M. Hable, Miss E. J. Nairne, and Mr. H. L. Gilliard, all of the Irrigation Research Station, C.S.I.R.O., Griffith, for their technical assistance.

VI. References

Best, R. J. (1950).—A rapid and accurate electro-titrimetric method for determining the chloride content of soils, water and plant materials. Trans. 4th Int. Congr. Soil Sci., Amsterdam. Vol. 3. pp. 162–5.

Briggs, G. E., and Robertson, R. N. (1957).—Apparent free space. *Annu. Rev. Pl. Physiol.* **8**: 11–30.

Chapman, H. D. (1949).—Citrus leaf analysis. *Calif. Agric.* **3**(11): 10–12, 14.

Cooper, W. C., Gorton, B. S., and Olson, E. O. (1952).—Ionic accumulation in citrus as influenced by root stock, scion and concentration of salts and boron in the substrate. *Plant Physiol.* **27**: 191–203.

Epstein, E. (1956).—Passive permeation and active transport of ions in plant roots. U.S. Atomic Energy Comm. TID-7512. pp. 297–301.

Gauch, H. G. (1957).—Mineral nutrition of plants. *Annu. Rev. Pl. Physiol.* **8**: 31–64.

Harding, R. B., Miller, M. P., and Fireman, M. (1957).—Leaf burn on sprinkled citrus. *Calif. Agric.* **11**(1): 9–10.

Helder, R. J. (1952).—Analysis of the process of anion uptake of intact maize plants. *Acta Bot. Neerl.* **1**: 361–434.

Hylmö, B. (1953).—Transpiration and ion absorption. *Physiol. Plant.* **6**: 333–405.

Long, E. M. (1943).—The effect of salt additions to the substrate on intake of water and nutrients by roots of approach-grafted tomato plants. *Amer. J. Bot.* **30**: 594–601.

Phillis, E., and Mason, T. G. (1942).—On diurnal variations in the mineral content of the leaf of the cotton plant. *Ann. Bot., Lond.* (N.S.) **6**: 437–42.

STEWARD, F. C., PREVOT, P., and HARRISON, Y. A. (1942).—Absorption and accumulation of rubidium bromide by barley plants. Localization in the root of cation accumulation and of transfer to the shoot. *Plant Physiol.* **17**: 411–21.

ULRICH, A., and OHKI, K. (1956).—Chlorine, bromine and sodium as nutrients for sugar-beet plants. *Plant Physiol.* **31**: 171–81.

WIERSUM, L. K. (1947).—Transfer of solutes across the young root. *Rec. Trav. Bot. Neerl.* **41**: 1–79.

WILLIAMS, R. F. (1955).—Redistribution of mineral elements during development. *Annu. Rev. Pl. Physiol.* **6**: 25–42.

WOOLLEY, J. T., BROYER, T. C., and JOHNSTON, G. V. (1958).—Movement of chlorine within plants. *Plant Physiol.* **33**: 1–7.

CALCIUM IN THE NITROGEN METABOLISM OF SUBTERRANEAN CLOVER

By J. F. Loneragan*

[*Manuscript received October* 2, 1958]

Summary

It has been shown that calcium has a specific effect upon nitrogen fixation by subterranean clover (*Trifolium subterraneum* L.).

Under conditions of moderate calcium deficiency the yield of plants was low, percentage protein and percentage non-protein nitrogen were reduced, and the plants were pale green. Addition of either combined nitrogen or calcium caused the plants to become dark green and increased the yield, the percentage protein, and the percentage non-protein nitrogen. Molybdenum deficiency produced similar effects on yield, protein, and non-protein nitrogen as moderate calcium deficiency.

The dry weight of nodules was decreased by calcium deficiency. The nodules of pale green calcium-deficient plants also showed signs of degeneration while those of molybdenum-deficient plants were not affected. The percentage calcium content of the nodules of the pale green calcium-deficient plants was much less than that of the tops and, unlike the tops, did not increase with calcium treatment. The effects of calcium deficiency on nitrogen fixation were therefore not necessarily brought about by a shortage of calcium in the nodules, but may have been due to a restricted supply of metabolite, possibly carbohydrate, resulting from calcium deficiency in the host plant.

Under conditions of severe calcium deficiency, growth of the host plant was stunted by calcium deficiency rather than by nitrogen deficiency. The host plant contained a higher than normal percentage of nitrogen and did not respond to nitrogen fertilizer.

I. Introduction

The level of calcium in the plant is known to affect nitrogen fixation in legumes indirectly through its effect on plant growth (Horner 1936; Albrecht 1937). Calcium level is also known to affect nodulation (Albrecht and Davis 1929; Loneragan and Dowling 1958). There is, however, no evidence in the literature that calcium has a specific effect upon nitrogen fixation by legumes. This paper presents evidence that calcium has a specific effect upon nitrogen fixation in subterranean clover.

II. Methods

(*a*) *General*

Plants were grown on calcium-deficient soils in pots in a glass-house. The soils used were:

Soil A: a light brown sandy clay from Black Mountain, Canberra, of pH 5·6, exchange capacity 5·4 m-equiv/100 g, calcium <0·05 m-equiv/100 g.

Soil B: a light brownish grey loamy sand derived from Hawkesbury Sandstone, near Robertson, N.S.W., designated "Birrilee sand" by Walker (1959), of pH 5·0, exchange capacity 5·4 m-equiv/100 g, calcium <0·05 m-equiv/100 g.

---

*Division of Plant Industry, C.S.I.R.O., Canberra.

"Bacchus Marsh" strain of subterranean clover (*Trifolium subterraneum* L.) was used in experiments 1, 2, and 4. Wimmera rye grass (*Lolium rigidum* Gaud.) was used in experiment 3. Ten plants were grown per pot in all experiments.

The clover seed was inoculated with a dense suspension of *Rhizobium trifolii* strain NA 30 immediately before sowing. Four seeds were planted in each of 10 holes. Two milligrams of a mixture of calcium carbonate (1 part by weight) and the soil used in the experiment (9 parts) were placed on the seed before covering with soil. In this way nodules were formed on every plant of each treatment despite the acidity and the low calcium status of the soils.

In experiments 1, 2, and 3, 1900 g of soil A was used in glazed earthenware pots of $4\frac{5}{8}$ in. diameter. In experiment 4, 1120 g of soil B was used in enamel pots of the same diameter.

Basal dressings (expressed in weight per acre on a surface area basis) common to all experiments were:

K_2HPO_4	Equivalent in phosphorus to 8 cwt of superphosphate per acre ($\equiv$563 mg per pot).
K_2SO_4	3 cwt per acre ($\equiv$408 mg per pot).
H_3BO_3	2 lb per acre ($\equiv$2·4 mg per pot).
$ZnSO_4.7H_2O$	7 lb per acre ($\equiv$8·5 mg per pot).

The basal dressings were applied in solution prior to sowing.

All plants were harvested before flowering. The tops were dried rapidly under forced draught at 50°C. Roots of subterranean clover plants were washed from the soil and kept at 5°C until the nodules had been examined. Nodules which formed on the first 5 cm of tap root were designated "crown nodules". The remaining nodules were designated "distal nodules". Crown nodules were designated as "degenerating" when they showed definite brown discoloration or appeared burst and flaccid.

(*b*) *Details of Experiments*

Experiment 1.—To test the effect of calcium on subterranean clover grown at different levels of sodium molybdate, in the presence and absence of ammonium nitrate. Soil A. Sown January 29, 1958, and harvested April 15, 1958. A $3 \times 3 \times 2$ factorial design of the following fertilizers was used:

$CaSO_4$	Equivalent in calcium to nil, 2, or 8 cwt of superphosphate per acre ($\equiv$0, 206, or 824 mg per pot mixed through dry soil prior to sowing).
Na_2MoO_4	Nil, 4, or 16 oz per acre ($\equiv$0, 0·3, or 1·2 mg per pot added in solution).
NH_4NO_3	Nil or 5 cwt per acre ($\equiv$0 or 515 mg per pot). Applied in solution at the rate of 1 cwt per acre per week from the beginning of the seventh week after sowing.

A further two treatments, one with and one without ammonium nitrate were included: in each case calcium was supplied as calcium sulphate plus calcium chloride, equivalent respectively to 2 plus 6 cwt of superphosphate per acre ($\equiv$206 mg $CaSO_4$ plus 507 mg $CaCl_2$ per pot). Replicates 4. Seven weeks after sowing, a dressing of H_3BO_3 at the rate of 2 lb per acre ($\equiv$2·4 mg per pot) was applied to two replicates of each treatment.

Experiment 2.—To test the effect of calcium chloride on subterranean clover grown in the presence and absence of ammonium nitrate. Soil A. Sown and harvested with experiment 1. Sodium molybdate at 4 oz per acre was applied to the pots of all treatments. A 2×2 factorial design, with four replicates, of the following fertilizers was used:

$CaCl_2$	Equivalent in calcium to 2 or 8 cwt of superphosphate per acre (≡169 or 676 mg per pot added in solution before sowing).
NH_4NO_3	Same as experiment 1.

Experiment 3.—To test the effect of calcium on Wimmera rye grass grown in the presence and absence of ammonium nitrate. Soil A. Sown June 3, 1958, and harvested July 22, 1958. Sodium molybdate at 4 oz per acre was applied to the pots of all treatments. A 3×2 factorial design, with four replicates, of the following fertilizers was used:

$CaSO_4$	Equivalent in calcium to nil, 2, or 8 cwt of superphosphate per acre (≡0, 206, or 824 mg per pot mixed through the dry soil prior to sowing).
NH_4NO_3	Nil or 3·75 cwt per acre (≡0 or 386 mg per pot). Applied in solution at weekly intervals in increasing rates from 2 weeks after sowing.

Experiment 4.—To test the effect of calcium on subterranean clover grown in the presence and absence of ammonium nitrate. Soil B. Sown on September 12, 1957, and harvested on November 13, 1957. The following additional basal fertilizers were applied to the pots of all treatments:

$CuSO_4.5H_2O$	7 lb per acre (≡ 8·5 mg per pot).
Na_2MoO_4	4 oz per acre (≡ 0·3 mg per pot).
$MgSO_4.7H_2O$	14 lb per acre (≡ 17 mg per pot).

A 3×2 factorial design, with four replicates, of the following fertilizers was used:

$CaSO_4$	Equivalent in calcium to nil, 2, or 8 cwt of superphosphate per acre (≡ 0, 206, or 824 mg per pot mixed through the dry soil before sowing).
NH_4NO_3	Nil or 4·5 cwt per acre (≡ 464 mg per pot). Applied in solution at weekly intervals in increasing rates from 2 weeks after sowing.

(c) *Analytical Techniques*

Plant tops which were dried rapidly at 50°C immediately after harvest were finely ground, dried overnight at 105°C, and cooled in a desiccator prior to chemical analysis.

Total nitrogen was determined on 0·2-g samples by the Kjeldahl digestion and steam-distillation procedures of McKenzie and Wallace (1954).

Protein nitrogen was determined on 0·2-g samples after extraction of the non-protein nitrogen with 100 per cent. ethanol and 70 per cent. ethanol (three times) on a water-bath at 60°C. Each extraction was carried out for 15 min. For the extraction the plant material was wrapped in filter paper: nitrogen was determined on the plant residue plus filter paper as described for total nitrogen.

Non-protein nitrogen was calculated by difference between the total nitrogen and protein nitrogen fractions.

Calcium was determined on 0·2-g samples in an "E.E.L." flame photometer after ashing at 550°C and removal of interfering anions by exchange on chloride-saturated "Amberlite IR4B" resin.

III. Results

(*a*) *Yield and Nitrogen Content of Legumes*

(i) *Effects of Calcium.*—At lowest calcium levels plants exhibited acute calcium-deficiency symptoms, identical in both the young leaves and the old leaves with those described by Millikan (1953) for Dwalganup variety of subterranean clover. The growth of these plants was restricted by calcium deficiency and not by nitrogen deficiency. The plants did not respond to combined nitrogen treatment (Fig. 1). Also the percentage protein and percentage non-protein nitrogen were higher in these calcium-deficient than in the calcium-sufficient plants (Fig. 2).

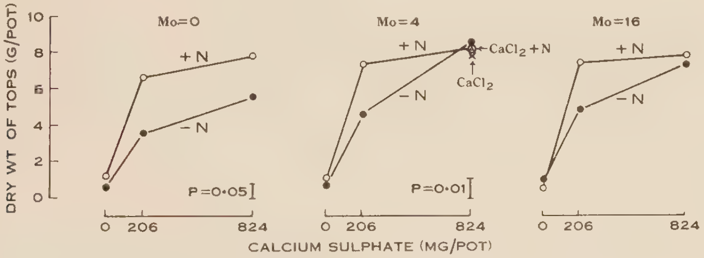


Fig. 1.—Effects and interactions of ammonium nitrate and calcium sulphate on yield of the tops of subterranean clover supplied with sodium molybdate at 0, 4, and 16 oz per acre. The values obtained where calcium chloride was added in addition to calcium sulphate are also given (expt. 1).

In plants which were moderately calcium deficient, calcium had a specific effect upon nitrogen fixation (Figs. 1, 2, and 3). Subterranean clover plants not provided with ammonium nitrate showed typical symptoms of nitrogen deficiency (pale green to yellow leaves with red petioles) with no symptoms of calcium deficiency. Percentage protein and percentage non-protein nitrogen were lowered and yield was reduced. Addition of ammonium nitrate restored to normal the percentage protein, percentage non-protein nitrogen, colour, and the yield: symptoms of calcium deficiency appeared in some of the older leaves (red leaves with withered petioles). The interaction between ammonium nitrate and calcium sulphate was strongly negative. Each fertilizer replaced the other in its effects on yield, colour, percentage protein, and percentage non-protein nitrogen (negative interaction significant for all effects at 5 per cent. level at least). This effect of calcium sulphate was shown to be due to the calcium ion. Calcium chloride was as effective as calcium sulphate in replacing ammonium nitrate in its effects on yield, colour, percentage protein, and percentage non-protein nitrogen (Figs. 1 and 2; Table 1).

The increase in percentage protein and percentage non-protein nitrogen of the severely calcium-deficient plants to which no combined nitrogen was added indicates that calcium became limiting to growth before soil nitrogen was exhausted.

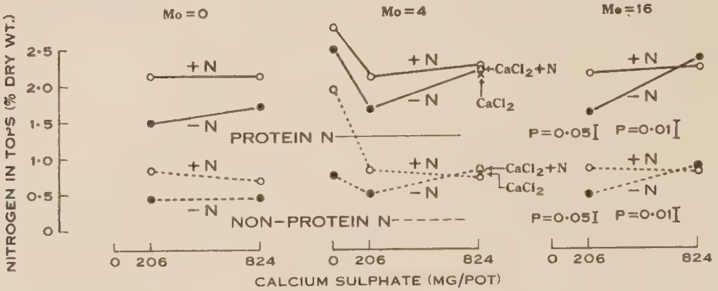


Fig. 2.—Effects and interactions of ammonium nitrate and calcium sulphate on percentage protein and percentage non-protein nitrogen of the tops of subterranean clover supplied with sodium molybdate at 0, 4, and 16 oz per acre. The values obtained where calcium chloride was added in addition to calcium sulphate are also given (expt. 1).

(ii) *Effects of Molybdenum.*—Molybdenum, like calcium, gave a negative interaction with combined nitrogen (significant at the 5 per cent. level at least) on yield, colour, percentage protein, and percentage non-protein nitrogen (Figs. 1 and 2).

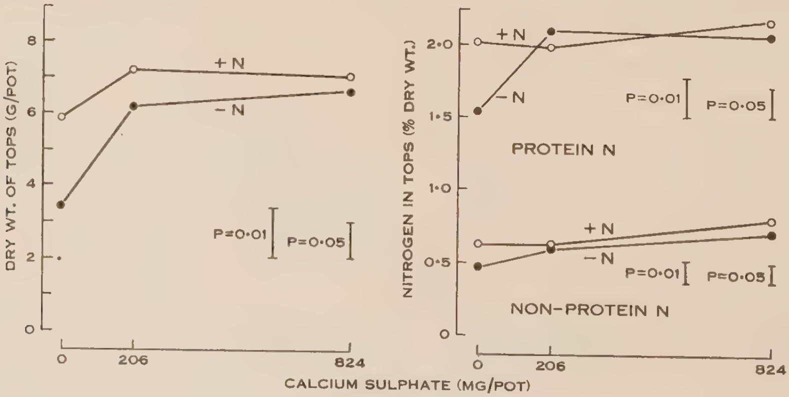


Fig. 3.—Effects and interactions of ammonium nitrate and calcium sulphate on yield, percentage protein, and percentage non-protein nitrogen of the tops of subterranean clover (expt. 4).

In the absence of ammonium nitrate, plants given no molybdenum and inadequate calcium were similar in colour, yield, and nitrogen content to plants deficient in only one of these elements. Dressings of ammonium nitrate alone produced dark green plants, and increased the yield, percentage protein, and percentage non-protein nitrogen to normal levels. However, where no combined nitrogen was applied, dressings of both molybdenum and calcium were required to give maximum increase in colour, yield, or percentage nitrogen. Thus molybdenum and calcium were both separately required for nitrogen fixation by subterranean clover.

(iii) *Effects of Boron.*—The direct effect of calcium on nitrogen fixation cannot be explained through any effect of calcium on the uptake or utilization of boron.

Table 1

EFFECTS OF AMMONIUM NITRATE AND CALCIUM CHLORIDE ON THE YIELD, PERCENTAGE PROTEIN NITROGEN, AND PERCENTAGE TOTAL NITROGEN OF THE TOPS OF SUBTERRANEAN CLOVER (EXPERIMENT 2)

	Without Ammonium Nitrate		With Ammonium Nitrate	
Calcium chloride (mg/pot):	169	676	169	676
Dry matter (g/pot)*	6·4	8·1	8·1	7·7
Total nitrogen (per cent. dry wt.)†	2·72	3·10	3·02	3·05
Protein nitrogen (per cent. dry wt.)‡	2·05	2·28	2·21	2·21

*Significant difference for $P = 0{\cdot}05$, 0·8; for $P = 0{\cdot}01$, 1·1. Interaction of calcium and nitrogen significant at $P < 0{\cdot}01$.

†Significant difference for $P = 0{\cdot}05$, 0·23; for $P = 0{\cdot}01$, 0·32. Interaction of calcium and nitrogen significant at $P < 0{\cdot}05$.

‡Significant difference for $P = 0{\cdot}05$, 0·20; for $P = 0{\cdot}01$, 0·26. Interaction of calcium and nitrogen not significant.

An application of boron (experiment 1) had no effect on yield, colour, percentage protein, percentage non-protein nitrogen, or even total nitrogen.

Table 2

EFFECTS OF AMMONIUM NITRATE AND CALCIUM SULPHATE UPON THE YIELD OF TOPS OF WIMMERA RYE GRASS (EXPERIMENT 3)

Results given as grams dry matter per pot. Mean log transformations given in parenthesis. Significant differences at $P = 0{\cdot}05$ and $P = 0{\cdot}01$ are 0·10 and 0·14 respectively (log transformations). Interaction of calcium and nitrogen significant at $P < 0{\cdot}001$

Calcium sulphate (mg/pot):	0	206	824
Without ammonium nitrate	0·27 (0·42)	0·36 (0·56)	0·45 (0·65)
With ammonium nitrate	0·56 (0·74)	2·99 (1·47)	3·12 (1·49)

(*b*) *Yield of Non-legumes*

The stimulating effects of calcium on the nitrogen content and growth of subterranean clover cannot be explained by an effect of calcium upon soil nitrogen. Wimmera rye grass responded markedly to combined nitrogen in the presence of adequate calcium (Table 2).

Where neither calcium nor ammonium nitrate was applied, plants showed a marked withering of the tips of the leaves. These symptoms were very similar to those of nitrogen deficiency except that a few of the withered tips showed a characteristic sickle-shaped curling. In the presence of ammonium nitrate the withering of the tip and the sickle-shaped curling were also present. In addition, at the junction of the withered tip and the green blade, the green leaf often exhibited a peculiar and marked puckering for a short distance.

(c) *Calcium Content of the Tops of Subterranean Clover*

Calcium content of the tops of subterranean clover plants increased markedly with increasing calcium supply (Table 3). Tops containing 0·08–0·09 per cent. of calcium showed severe symptoms of calcium deficiency. In the absence of combined nitrogen, plants containing 0·25–0·34 per cent. calcium showed symptoms of nitrogen deficiency and not of calcium deficiency. In some cases the application of combined nitrogen to these plants caused small increases in the concentration of calcium in the tops: the tops became green and showed slight but definite symptoms of calcium deficiency. Since this application of combined nitrogen was associated with an increase in yield there was an appreciable increase in the total calcium content of the tops. Only those plants containing 0·57–0·86 per cent. calcium in the tops were free of either nitrogen- or calcium-deficiency symptoms.

Table 3

EFFECTS OF AMMONIUM NITRATE, CALCIUM SULPHATE, AND CALCIUM CHLORIDE UPON THE CALCIUM CONTENT OF THE TOPS OF SUBTERRANEAN CLOVER (EXPERIMENTS 1, 2, AND 4)

Calcium content expressed as per cent. dry weight. Mean log transformations given in parenthesis

	Expt. No.	Calcium Salt	Without Ammonium Nitrate			With Ammonium Nitrate		
			Calcium (mg/pot)			Calcium (mg/pot)		
			0	60	240	0	60	240
Soil A*	1	Sulphate	0·09	0·25	0·64	0·08	0·32	0·73
	2	Chloride		0·28	0·71		0·34	0·82
Soil B†	4	Sulphate	0·34 (53)	0·57 (76)	0·85 (93)	0·33 (52)	0·62 (79)	0·86 (93)

*Significant difference for $P = 0\cdot05$, 0·11; for $P = 0\cdot01$, 0·15. Effect of nitrogen treatment at calcium levels 60 and 240 mg/pot significant at $P < 0\cdot05$.

†Significant difference (log transformation) for $P = 0\cdot05$, 9; for $P = 0\cdot01$, 12.

The increase in the total amount of calcium in the tops may have accounted in part for the stimulating effect of combined nitrogen on yield but could not account for the stimulation in protein content of moderately calcium-deficient plants. Where combined nitrogen was applied calcium deficiency did not reduce percentage protein despite the presence of definite calcium-deficiency symptoms and, in some cases, depression in yield. In fact, severe calcium deficiency even increased the percentage protein while at the same time it lowered the percentage calcium of the tops to one-third its level in the moderately calcium-deficient plants. The stimulating effect of ammonium nitrate on the percentage protein of moderately calcium-deficient plants must therefore have been due to the increased supply of nitrogen available for metabolism. This indicates that calcium functions specifically in the fixation of nitrogen.

Table 4

EFFECTS OF AMMONIUM NITRATE, CALCIUM SULPHATE, AND SODIUM MOLYBDATE UPON THE NODULES OF SUBTERRANEAN CLOVER (EXPERIMENT 1)

Mean log transformations shown in parenthesis; — = not determined

Nodules Measured	Sodium Molybdate (oz/ac)	Without Ammonium Nitrate			With Ammonium Nitrate		
		Calcium Sulphate (mg/pot)			Calcium Sulphate (mg/pot)		
		0	206	824	0	206	824
Distal (No./pot)*	0	0	81 (1·87)	105 (1·99)	2	16 (1·18)	17 (1·23)
	4	1	98 (1·97)	28 (1·43)	2	9 (0·91)	16 (1·18)
	16	2	76 (1·85)	22 (1·33)	1	12 (1·05)	10 (0·96)
Crown (No./pot)†	0	10 (0·95)	76 (1·88)	63 (1·80)	15 (1·17)	44 (1·64)	44 (1·63)
	4	17 (1·20)	70 (1·84)	55 (1·73)	12 (1·08)	45 (1·64)	40 (1·60)
	16	23 (1·30)	77 (1·88)	44 (1·62)	8 (0·81)	48 (1·67)	39 (1·58)
Crown (mg/pot)‡	0	—	122	155	—	—	—
	4	—	106	176	—	50	59
	16	—	96	134	—	—	—
Crown (mg/nodule)§	0	—	1·59	2·43	—	—	—
	4	—	1·51	3·20	—	1·01	1·50
	16	—	1·29	3·27	—	—	—
Crown (No. degenerating/pot)‖	0	3 (0·44)	27 (1·32)	0	8 (0·93)	6 (0·62)	0
	4	10 (0·99)	46 (1·67)	3	5 (0·65)	11 (0·81)	1
	16	14 (1·01)	52 (1·70)	2	6 (0·81)	9 (0·74)	6

*Significant difference (log transformation) for *P* = 0·05, 0·28; for *P* = 0·01, 0·37.

†Significant difference (log transformation) for *P* = 0·05, 0·19; for *P* = 0·01, 0·26.

‡Significant difference for *P* = 0·05, 44; for *P* = 0·01, 60.

§Significant difference for *P* = 0·05, 0·81; for *P* = 0·01, 1·10.

‖Significant difference (log transformation) for *P* = 0·05, 0·55; for *P* = 0·01, 0·73.

(*d*) *Nodulation*

(i) *Nodule Numbers.*—Those treatments which reduced the percentage nitrogen content of the tops increased the number of nodules on the roots (Table 4). Moderate calcium deficiency and molybdenum deficiency, separately or together, decreased nitrogen content and increased nodule numbers. This suggests that the effect of moderate calcium deficiency in promoting nodule formation was a secondary effect of induced nitrogen deficiency. The marked reduction in nodule numbers by the addition of ammonium nitrate in all treatments supports this view.

(ii) *Nodule Weight.*—The dry weight of the crown nodules was reduced by moderate calcium deficiency (Table 4). Molybdenum deficiency had no effect on the total dry weight of the crown nodules, but, through its effect on nodule numbers, decreased the dry weight per nodule.

(iii) *Nodule Degeneration.*—Calcium deficiency had a specific and marked effect upon nodule degeneration (Table 4). This degeneration varied from brown discoloration to a burst, deflated appearance of the nodules. This was not a secondary effect of nitrogen deficiency since the proportion of degenerating crown nodules on severely calcium-deficient plants containing a high percentage nitrogen was as high as on moderately calcium-deficient plants of low percentage nitrogen. Moreover, plants suffering from nitrogen deficiency induced by lack of molybdenum showed no nodule degeneration.

Table 5

EFFECTS OF AMMONIUM NITRATE AND CALCIUM SULPHATE UPON THE PERCENTAGE CALCIUM CONTENT OF THE NODULES OF SUBTERRANEAN CLOVER (EXPERIMENT 1)

Calcium content given as per cent. dry weight. Differences not significant

Calcium sulphate (mg/pot):	206	824
Without ammonium nitrate	0·16	0·17
With ammonium nitrate	0·16	0·20

(iv) *Calcium Content of Nodules.*—Moderate calcium deficiency had no significant effect on the percentage calcium content of the crown nodules (Table 5). This lack of response to calcium treatment is in striking contrast to the effects of these treatments on the calcium content of the tops. It suggests that calcium might not function in nitrogen fixation in the nodule itself but rather through the supply of metabolites from the plant tops.

IV. Discussion

Under conditions of extreme calcium deficiency, growth of subterranean clover plants was stunted by calcium deficiency rather than by nitrogen deficiency. The host plant contained higher than normal percentage nitrogen and did not respond to nitrogen fertilizer. These findings are in keeping with the results of Horner (1936) and Albrecht (1937) who found that the total amount of nitrogen fixed by soybean plants decreased with decreasing concentrations of calcium in the plant: as calcium decreased, percentage nitrogen in the plants increased, so that the effect of calcium on the amount of nitrogen fixed was through its effect on growth of the host plant.

Under conditions of moderate calcium deficiency, nitrogen fixation was specifically inhibited. The yield of plants was low, percentage protein and percentage non-protein nitrogen were reduced, and the plants were pale green. Addition of ammonium nitrate caused the plants to become dark green and increased both the percentage nitrogen content and the yield of the plants. In these respects ammonium nitrate replaced additional dressings of calcium.

Molybdenum deficiency affected nitrogen metabolism of the tops of subterranean clover in a similar manner to moderate calcium deficiency. The effects of molybdenum on the nitrogen metabolism of subterranean clover have been described previously (Anderson and Thomas 1946; Anderson and Spencer 1950*a*). Molybdenum deficiency decreased both the percentage and the total amount of nitrogen in subterranean clover plants. The percentage protein and the percentage non-protein nitrogen were each decreased by deficiency of molybdenum. Addition of combined nitrogen to molybdenum-deficient plants gave dark green plants, yield increases, and increases in both percentage protein and percentage non-protein nitrogen. Under some conditions, addition of nitrate nitrogen produced these effects concurrently with a decrease in both percentage and total molybdenum contents of the tops of the plants (Anderson and Spencer 1950*a*). Thus it was established conclusively that, in the soils used, molybdenum was required only for the fixation of nitrogen.

Sulphur deficiency of subterranean clover produced pale green plants indistinguishable in appearance and in total nitrogen content from molybdenum-deficient plants (Anderson and Spencer 1950*b*). However, sulphur deficiency differed from molybdenum deficiency in that although it decreased percentage total and percentage protein nitrogen, it increased percentage non-protein nitrogen. Addition of sulphur increased the percentage protein nitrogen but decreased the percentage non-protein nitrogen. Furthermore, although addition of combined nitrogen increased the percentage total nitrogen it did not affect the colour or the percentage protein nitrogen Thus sulphur was required for the conversion of non-protein into protein nitrogen so that the inhibition of nitrogen fixation by sulphur deficiency was caused by the non-utilization of the nitrogen fixed.

The effects of moderate calcium deficiency and its interactions with combined nitrogen on percentage protein and percentage non-protein nitrogen of subterranean clover were similar to those of molybdenum and quite distinct from those of sulphur deficiency. Calcium then did not affect conversion of non-protein to protein nitrogen. An effect of calcium upon the metabolism of the first-formed products of nitrogen fixation could conceivably lead to the observed low protein and non-protein nitrogen contents in moderately calcium-deficient plants. Increasing the supply of combined nitrogen did, however, replace dressings of calcium in restoring the percentage protein and non-protein nitrogen to normal. Nevertheless, this evidence does not establish direct participation of calcium in nitrogen fixation since the dressings of combined nitrogen increased the amount (although not always the percentage) of calcium in the tops of the plants. However, if calcium is directly concerned in the metabolism of non-protein nitrogen, calcium deficiency would lead to low percentage protein in plants supplied with combined nitrogen. There was no evidence of any inhibition of protein synthesis where combined nitrogen was supplied, even in plants showing definite symptoms of calcium deficiency. There was, in fact, a stimulation in percentage protein in those plants showing the most severe symptoms of calcium deficiency and with the lowest percentage of calcium in the tops. Furthermore, the failure of calcium deficiency to inhibit protein formation has been reported for many plants grown in the presence of combined nitrogen (Ginsberg and Shive 1926; Nightingale *et al.* 1931; Hibbard and Grigsby 1934). The low percentage protein of 3-day-old calcium-deficient wheat seedlings observed by Burström (1954) was due almost entirely to inhibition of nitrate uptake. The present results cannot be explained by any effect of calcium deficiency on nitrogen uptake. The evidence therefore suggests that calcium functions specifically in nitrogen fixation in subterranean clover.

Effects of boron deficiency in legumes (Brenchley and Thornton 1925; Mulder 1948) are in some respects similar to the effects of moderate calcium deficiency and of molybdenum deficiency. Boron deficiency reduced nitrogen fixation in *Vicia faba* (Brenchley and Thornton 1925). Percentage nitrogen was reduced and the plants showed symptoms of nitrogen deficiency. However, the plants showed symptoms of boron deficiency together with those of nitrogen deficiency, and did not respond much in yield to addition of combined nitrogen unless boron was also added. The effect of boron on nitrogen fixation was therefore largely due to its effect on the growth of the host plant. Mulder (1948) obtained similar results with pea plants grown in water culture.

Nitrogen deficiency induced by low calcium or molybdenum supply was associated with an increase in the number of nodules. Application of nitrogen to plants deficient in either calcium or molybdenum increased the percentage nitrogen and decreased the number of nodules. The same effect was obtained also where the plants were deficient in both calcium and molybdenum. Effects of molybdenum deficiency on nodulation were reported and discussed fully by Anderson and Spencer (1950*a*). By contrast with molybdenum, calcium, and nitrogen deficiencies, which decreased the percentage nitrogen and increased the number of nodules, sulphur deficiency reduced both the percentage nitrogen content of subterranean clover plants and the nodule numbers (Anderson and Spencer 1950*b*). This result led Anderson and Spencer to reject the carbon–nitrogen hypothesis (Wilson 1940) as an explanation of the effects of sulphur on nodulation. They pointed out that in all cases investigated by them, decrease in the number of nodules was associated with an increase in the percentage non-protein nitrogen. The increase in nodule numbers in moderately calcium-deficient plants reported in the present experiments was also accompanied by a decrease in the percentage non-protein nitrogen.

Anderson and Spencer (1950*b*) also drew attention to a correlation between nodule numbers and nitrogen requirement of the host plant: response to nitrogen was accompanied by decrease in the number of nodules. The effect of moderate calcium deficiency in increasing nodule numbers is consistent with the claim that growth of these plants was limited by supply of available nitrogen and that calcium functions specifically in nitrogen fixation.

The increase in nodule numbers associated with calcium-deficient plants of low nitrogen status was therefore a secondary and not a direct effect of the calcium deficiency. In fact, where secondary effects of plant growth and nitrogen deficiency were eliminated by supply of adequate combined nitrogen, decreasing calcium supply decreased the number of nodules on young subterranean clover plants (Loneragan and Dowling 1958). Calcium supply had no effect on the growth of *Rhizobium* so that the extra calcium was required for infection or nodule development. The premature degeneration of the nodules of plants grown with low calcium in the present experiments indicates that the extra calcium was required for nodule development.

The degeneration of nodules in calcium-deficient plants is not a secondary effect of nitrogen deficiency. Even where ammonium nitrate was applied the number of degenerating nodules was higher on calcium-deficient than on calcium-sufficient plants. Moreover, the high percentage of degenerating nodules present on the crowns of moderately calcium-deficient plants is in striking contrast to their complete absence on molybdenum-deficient plants. In addition, there was a very high proportion of degenerating nodules on the severely calcium-deficient plants which showed no signs of nitrogen deficiency. This suggest that calcium deficiency induced premature nodule degeneration before it had any effect on nitrogen fixation.

The low calcium content of the nodules compared with that of the tops suggests that the nodules have a lower overall requirement for calcium. Jensen (1947) has also reported lower calcium levels in the nodules of several legumes, including subterranean clover, than in the tops. It is possible that a specific part of the nodule has a high calcium requirement. However, it seems unlikely that nodule development was directly influenced by the level of calcium in the nodule, since there was no difference between the percentage calcium contents of the nodules of the intermediate and high calcium treatments of the present experiments.

On the other hand, calcium could affect nitrogen fixation in the nodules through an effect on the supply of metabolites from the roots or tops. It has been suggested (Brenchley and Thornton 1925) that boron deficiency affects nitrogen fixation in this way. Under conditions of boron deficiency vascular strands failed to develop in the nodules of *Vicia faba* plants and nitrogen fixation was reduced. Nodules of plants grown without boron presented a curious burst appearance. The degeneration of nodular tissue of plants without boron was attributed by these authors to an inadequate carbohydrate supply. Mulder (1948) also reported that the nodules of pea plants not given boron and showing symptoms of nitrogen deficiency were black and shrivelled at harvest.

The similarity in the effects of calcium and boron deficiencies on nodule degeneration suggests that calcium may also be involved in supply of carbohydrates to the nodule.

There is also a strong similarity between the effects of low calcium supply on the nodules of subterranean clover plants and of reduced carbohydrate supply on the nodules of lucerne plants placed in the dark (Thornton 1930). In darkened lucerne plants, as in low calcium subterranean clover plants (Loneragan and Dowling 1958) new nodules were not formed and existing nodules degenerated prematurely.

There is abundant evidence that calcium deficiency interferes with carbohydrate metabolism. According to Miller (1938), Boehm as early as 1875 observed an abnormal accumulation of starch in *Phaseolus vulgaris* grown without calcium; this has been confirmed for numerous plants by many investigators. In the same experiments in which organic nitrogen was not affected, Nightingale *et al.* (1931) and Hibbard and Grigsby (1934) found marked accumulation of carbohydrates in calcium-deficient tomato stems and pea plants. Joham (1957) found an accumulation of carbohydrates in the tops and depletion in the roots of calcium-deficient cotton plants, indicating that calcium has a role in the translocation of carbohydrates. The evidence therefore suggests that the premature degeneration of the nodules of calcium-deficient plants may well have resulted from such an effect of calcium deficiency on translocation of carbohydrates to the nodule. The effect of calcium deficiency on nitrogen fixation may also have resulted from its effects on the supply of carbohydrates or some other metabolite to the nodule.

Of the many species and strains of *Azotobacter* investigated, only one has been shown to have a specific requirement for calcium in nitrogen fixation (Burk and Horner 1939; Esposito and Wilson 1956; Norris and Jensen 1957). Allen and Arnon (1955) found no difference in the calcium requirement between plants of the blue-green alga *Anabaena cylindrica* grown on molecular nitrogen and on nitrate. These results are compatible with the suggestion that calcium affects nitrogen fixation by subterranean clover through its effect on the supply of metabolites to the nodule rather than through direct participation in the biochemical reactions of fixation.

V. Acknowledgments

The author is indebted to Mr. G. Dudzinski, Division of Mathematical Statistics, C.S.I.R.O., for statistical treatment of the data, to Mr. P. H. Walker for supplying the "Birrilee Sand", to Mr. E. J. Dowling for technical assistance, and to Dr. A. J. Anderson for stimulating discussion and advice.

VI. References

Albrecht, W. A. (1937).—Physiology of root nodule bacteria in relation to fertility levels of the soil. *Proc. Soil Sci. Soc. Amer.* **2**: 315–27.

Albrecht, W. A., and Davis, F. L. (1929).—Physiological importance of calcium in legume inoculation. *Bot. Gaz.* **88**: 310–21.

Allen, M. B., and Arnon, D. I. (1955).—Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *Plant Physiol.* **30**: 366–72.

Anderson, A. J., and Spencer, D. (1950*a*).—Molybdenum in nitrogen metabolism of legumes and non-legumes. *Aust. J. Sci. Res.* B **3**: 414–30.

Anderson, A. J., and Spencer, D. (1950*b*).—Sulphur in nitrogen metabolism of legumes and non-legumes. *Aust. J. Sci. Res.* B **3**: 431–49.

Anderson, A. J., and Thomas, M. P. (1946).—Plant responses to molybdenum as a fertilizer. 1. Molybdenum and symbiotic nitrogen fixation. Bull. Coun. Sci. Industr. Res. Aust. No. 188. Pt. I.

Brenchley, W. A., and Thornton, H. G. (1925).—The relation between the development, structure and functioning of the nodules on *Vicia faba*, as influenced by the presence or absence of boron in the nutrient medium. *Proc. Roy. Soc.* B **98**: 373–99.

Burk, D., and Horner, C. K. (1939).—Molybdenum and calcium in *Azotobacter* nutrition. Proc. 3rd Int. Congr. Microbiol., New York. pp. 489–90.

Burström, H. (1954).—Studies on growth and metabolism of roots. X. Investigations of the calcium effect. *Physiol. Plant.* **7**: 332–42.

Esposito, R. G., and Wilson, P. W. (1956).—Trace metal requirements of *Azotobacter*. *Proc. Soc. Exp. Biol., N.Y.* **93**: 564–7.

Ginsburg, J. M., and Shive, J. W. (1926).—The influence of calcium and nitrogen on the protein content of the soybean plant. *Soil Sci.* **22**: 175–97.

Hibbard, R. P., and Grigsby, B. H. (1934).—Relation of light, potassium, and calcium deficiencies to photosynthesis, protein synthesis and translocation. Tech. Bull. Mich. Agric. Exp. Sta. No. 141.

Horner, G. M. (1936).—Relation of the degree of base saturation of a colloidal clay by calcium to the growth, nodulation and composition of soybeans. Res. Bull. Miss. Agric. Exp. Sta. No. 232.

Jensen, H. L. (1947).—The calcium content of legume root nodules. *Proc. Linn. Soc. N.S.W.* **72**: 203–6.

Joham, H. E. (1957).—Carbohydrate distribution as affected by calcium deficiency in cotton. *Plant Physiol.* **32**: 113–17.

Loneragan, J. F., and Dowling, E. J. (1958).—The interaction of calcium and hydrogen ions in the nodulation of subterranean clover. *Aust. J. Agric. Res.* **9**: 464–72.

Miller, E. C. (1938).—"Plant Physiology." 2nd Ed. p. 312. (McGraw-Hill Book Co., Inc.: New York.)

Millikan, C. R. (1953).—Nutritional disorders in subterranean clover. Tech. Bull. Vict. Dep. Agric. No. 11.

Mulder, E. G. (1948).—Investigations on the nitrogen nutrition of pea plants. *Plant & Soil* **1**: 179–212.

McKenzie, H. A., and Wallace, H. S. (1954).—The Kjeldahl determination of nitrogen: a critical survey of digestion conditions—temperature, catalyst, and oxidizing agent. *Aust. J. Chem.* **7**: 55–70.

Nightingale, G. T., Addoms, R. M., Robbins, W. R., and Schermerhorn, L. G. (1931).—Effects of calcium deficiency on nitrate absorption and on metabolism in tomato. *Plant Physiol.* **6**: 605–30.

Norris, J. R., and Jensen, H. L. (1957).—Calcium requirements of *Azotobacter*. *Nature* **180**: 1493–4.

Thornton, H. G. (1930).—The influence of the host plant in inducing parasitism in lucerne and clover nodules. *Proc. Roy. Soc.* B **106**: 110–22.

Walker, P. H. (1959).—A reconnaissance of the soils and land use of part of the south coast of New South Wales. C.S.I.R.O. Aust. Soils and Land Use Ser. (in press.)

Wilson, P. W. (1940).—"The Biochemistry of Nitrogen Fixation." (University of Wisconsin Press: Madison.)

PHYSIOLOGY OF CEREAL GRAIN

I. THE SOURCE OF CARBON FOR THE DEVELOPING BARLEY KERNEL*

By M. S. Buttrose† and L. H. May†

[*Manuscript received September 22, 1958*]

Summary

Organic substrate for development of the barley kernel rises mainly from current assimilation of carbon dioxide. This paper describes a study of the relative importance of the several potential sources of substrate using ear shading, carbon-14 as tracer, and kernel-competition techniques. The application of the ear-shading technique to awnless, single-awned, and triple-awned varieties of barley led to significant reductions in yield of 9, 23, and 18 per cent. respectively, although the differences between the reductions themselves were not significant. Carbon-14 was supplied either to attached ears as $^{14}CO_2$ or to detached ears through the peduncle as ^{14}C-sucrose. Whichever source of carbon-14 was supplied, radioactivity was incorporated in the kernel from anthesis through to maturity, and its subsequent distribution in the kernel was similar. Translocation following $^{14}CO_2$ feeding was also examined, and it is concluded that little movement occurred except at an early stage of ear development. Competition between kernels was not evident, although it was induced when carbohydrate supply was artificially depleted. A scheme to accommodate the results is discussed, and a new method for calculating the maximum contribution of photosynthesis within the ear to kernel yield is presented.

I. Introduction

Archbold (1945) reviewed the endeavours of researchers over a half-century to define and evaluate the several sources of the carbohydrates that form the substrate for kernel development in the cereal ear. The conclusions reached were that material for endosperm development arose mainly from current carbon dioxide fixation in the upper leaf sheaths, the stem, and the ear itself, and that the contribution from sugar previously stored in the stem was small. More recently Porter, Pal, and Martin (1950) made the first direct measurement of assimilation and respiration of barley ears attached to the plant, and their results confirmed earlier estimates, involving shading techniques, that ear assimilation contributes about 30 per cent. of the final ear dry weight. For wheat, the ear's contribution has been calculated from ear-shading experiments to fall within the range 11–46 per cent. depending on season and variety (Asana and Mani 1950, 1955).

A feature of research in this field has been the wide range of the estimates of contributions from the several sources. This variability may stem not only from differences of environment and variety, but also from differences of technique. It has been generally recognized that the shading technique is open to serious criticism. Watson and Norman (1939) called attention to the alterations of temperature, rate of respiration, and rate of senescence which might be induced by shading. Again, Archbold and Datta (1944) concluded that secondary effects of shading were also at play affecting kernel yield by mechanisms other than the prevention of photosynthesis. One further criticism of the ear-shading technique, or any technique which removes one source of substrate, may be raised. This is to question the assumption, implicit in these techniques, that the various plant organs always contribute the same amount of carbon substrate to kernel development. It could equally be assumed that in the normal plant these organs do not contribute a maximum and that when one contributor is removed contributions from the remainder compensate at least in part for the deficiency.

---

*The data recorded in this part formed part of the thesis presented by one of us (M.S.B.) for the degree of M.Sc. in the University of Adelaide.

†Department of Plant Physiology, Waite Agricultural Research Institute, Adelaide.

In this work three approaches have been made to study further the role of photosynthesis in the ear, especially with a view to circumventing the difficulties inherent in shading techniques. Firstly, it is argued that ear shading in different varieties, similar except for their ear's photosynthetic surface area, should lead to reductions in yield in proportion to this area, providing that the only effect of ear shading is to prevent photosynthesis. This argument is supported by the observation of Vervelde (1953) that carbon dioxide assimilation in ears of barley, wheat, and rye respectively contributes to kernel development in this order of importance, which follows the order of the relative photosynthetic area of their ears. Secondly, radioactive carbon is used to examine, without shading, the ear's contribution to kernel yield and to follow the subsequent fate of the ear assimilate. Little previous work of this nature has been reported although some measurements of translocation of assimilate from leaves to the ears have been made (Fujiwara and Suzuki 1957; Kravcova 1957). These studies have indicated that only the uppermost leaves contribute to any marked extent. Thirdly, the proposition that plant organs may not normally contribute to their full potential is examined through a study of interkernel competition. Appleman and Eaton (1921) have noted that a change in kernel number per ear in maize had little effect on seed weight, and Donald (1954) found only relatively small effects in rye. However, information on this aspect of cereal physiology is scarce.

II. Materials and Methods

(a) *Barley Varieties*

In the ear-shading experiment three six-row varieties were selected for uniformity of vegetative characters and growing period; their major difference lay in the extent of awn development. One variety was awnless (*Hordeum vulgare* var. Arlington Awnless), the second had one awn per floret (*H. vulgare* var. Mars), and the third had three awns per floret (*H. vulgare* var. Long-awned Outer Glume). Assimilatory activities of normal ears from these varieties are different, and they have been estimated by measuring rates of carbon dioxide uptake under comparable conditions to lie in the proportion 3 : 7 : 10 respectively (Buttrose 1956).

In all other experiments a two-row barley (*H. distichum* var. Prior) was used. It is a single-awned local selection widely grown in South Australia.

(b) *Culture Techniques*

A sand-culture technique was used in much of this work. Enamel pots were filled with well-washed river sand (16·9 kg), and water added to bring to 65 per cent. of field capacity. Water additions were regulated by periodic weighings, and inorganic nutrients were added at weekly intervals between 2 and 10 weeks after planting.

In the remainder of the work plants were grown in "John Innes" potting compost in earthenware pots. Water additions were frequent so as to maintain the soil at field capacity.

(c) *Ear-shading Technique*

Opaque shades comprising two paper cylinders, one inside the other, were modelled on those described by Archbold (1942). Ventilation holes were provided in such a way that direct pathways for light entry were avoided, and the amount of light which penetrated to the inside, where the ear was housed, was very small. Individual ears were tagged at anthesis but shades were not fitted until 4 days later, otherwise pollination of the later-flowering spikelets may have been impaired.

(d) *Feeding Radioactive Sucrose* (*^{14}C-sucrose*)

An ear with a portion of stem was removed, and the stem recut twice beneath water to leave 3 cm below the basal floret. The attached part of the stem was placed in a small tube containing 3 per cent. ^{14}C-sucrose (1·5 μc in 1·5 ml). Feeding took place in a glass-house for 5 hr. Then the ear was transferred, after rinsing its stem, to distilled water and kernels were sampled at intervals over a period of 9 days.

(e) *Feeding Radioactive Carbon Dioxide* (*$^{14}CO_2$*)

(i) *To Whole of Attached Ears.*—A 1-cm strip of thin rubber sheeting was wrapped and fixed, under slight tension, round 3 cm of peduncle immediately below the ear. After this preparation, the ear, still attached to the potted plant, was introduced into the apparatus illustrated in Figure 1. The bound portion of the stem was inserted through a slit into a hole in a rubber stopper which, in turn, was secured in the mouth of a glass tube (12 by 1 in.). The outside of the stopper was heavily greased to make an air seal. The glass aspirator (*A*) contained water covered by a layer (0·5 cm) of paraffin oil (*D*). The rubber stopper in the mouth of this aspirator positioned a glass tube (*B*), which contained lactic acid (80 per cent.), so that its lower opening was inside a second tube (*C*) containing ^{14}C-barium carbonate (5 μc in 8·9 mg). Air (6 l.) was drawn through carbon dioxide-absorption towers (*E*) by draining the aspirator, and a negative pressure was established inside the aspirator sufficient to accommodate the $^{14}CO_2$ released when lactic acid was added to the barium carbonate. The concentration of carbon dioxide was 0·076 per cent. (v/v). When the gases inside the aspirator had equilibrated they were drawn over the ear by regulating a vacuum pump at one end of the gas line and the rate of water replacement to the aspirator from the reservoir (*F*) at the other. By suitable adjustment a steady flow rate (1·5 l/hr) was maintained at a slight negative pressure (1 cm of water, manometer *G*). It follows that feeding occupied 4 hr.

After treatment the ear was removed from the apparatus and the whole plant placed beneath a wire-netting cage on an open site. Kernels were harvested at intervals in the period to ear maturity.

(ii) *To Half of Attached Ears.*—To observe translocation within the ear either the top or bottom half of the ear was supplied with $^{14}CO_2$. Where the top half was exposed to $^{14}CO_2$, three florets on either side at the centre of a two-row ear were removed and the exposed rachis denuded of hairs and sterile spikelets. A narrow strip of rubber sheeting was bound round this part of the rachis and the top half of the ear inserted into the assimilation apparatus. Where the bottom half was fed $^{14}CO_2$, the whole ear was inserted into the assimilation apparatus, and light was excluded from the top half of the ear with an aluminium shade. Again six central florets were removed.

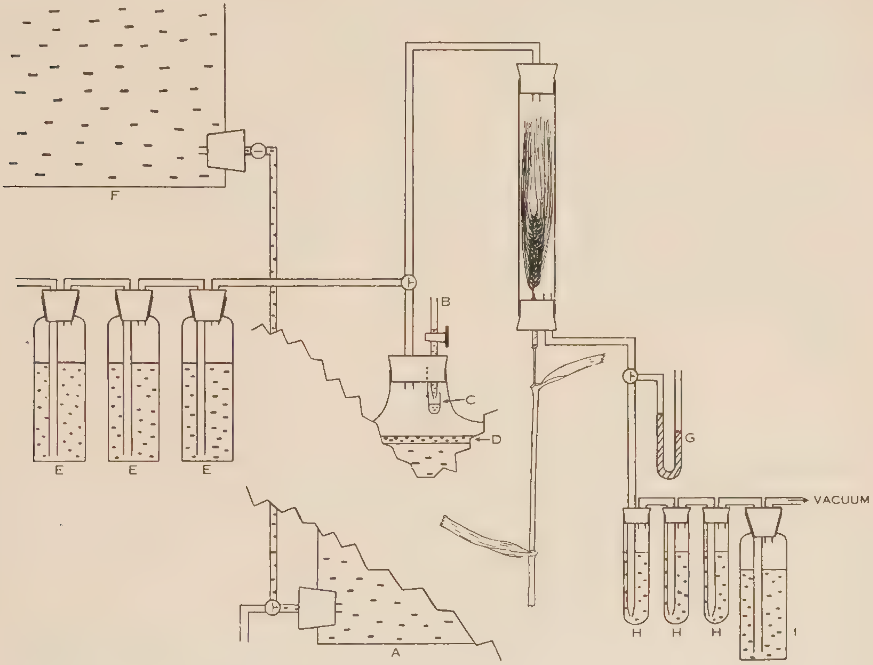


Fig. 1.—Apparatus used in feeding $^{14}CO_2$ to barley ears (see text).

(*f*) *Autoradiography*

Samples were stored in ethanol after harvest, and the ethanol was changed several times to remove soluble substances. Kernel sections were cut either transversely, midway between base and apex, or longitudinally, through the kernel "cheeks". The sections were washed with three changes of ethanol (80 per cent.), dried, and transferred to the photographic emulsion, close contact being maintained between section and emulsion. Two types of film were employed: $3\frac{1}{4}$ by $3\frac{1}{4}$ in. lantern-slide plates (Kodak Ltd.), and "Kodirex" X-ray film. When plates were used they were exposed for 28 days in the dark, developed in "D72" (Kodak developer) for 3 min at 23°C, fixed in acid fixer for 15 min, and washed. When X-ray film was used it was exposed for 7 days, developed for 2 min at 23°C with X-ray developer, and fixed. Plant material other than sections was examined for radioactivity in a similar way.

(g) *Reducing Kernel Numbers and Substrate Supply*

Treatments were carried out on ears (two-row) initially reduced to 18 florets, the number in excess being removed from the apex. Where a further six florets were removed those taken were 1, 4, and 7 on each side (counting from the base). Where 12 florets were removed those in positions 3, 6, and 9 were also taken. In another treatment kernel development was prevented in 12 florets without removing their glumes or awns by incising the outer glume and removing the ovule. Finally, defoliation and stem shading were superimposed on a reduction in kernel number. This was achieved by cutting the leaf blade at the junction with the leaf sheath, and loosely wrapping the stem with several layers of brown paper. The shades were extended where necessary to cover later stem growth. Treatments were imposed between 3 and 5 days after anthesis.

III. RESULTS

(a) *Effects of Ear Shading on Kernel Development*

Four seeds of awnless, single-awned, and triple-awned barley were planted in each of 15 pots of sand; 2 weeks later plants were thinned to six per pot (two per variety). In each pot all the ears on three plants, one of each variety, were shaded; the other three plants remained untreated. Ears were harvested when the peduncle was dry, threshed, and the kernels oven dried at 105°C.

The results (means of 15 plants) for the shaded and unshaded treatments are shown in Table 1. The mean yield per kernel for each variety was reduced by shading. However, the differences between the reductions themselves were not significant at the 5 per cent. level. The data collected in these experiments were very variable. Analysis of this variation showed it to be independent of treatment and pot, and suggested that the occurrence was at random. In this connexion great variation was also noted during growth; some plants within each variety were apparently quite healthy, while others in the same pot were not. It is concluded that the varieties used were unsuited to the environment imposed upon them since the Prior variety, grown under exactly the same conditions, did not show this variation.

(b) *The Fate of Radioactive Carbon* (*carbon*-14) *Supplied to the Ear either as Carbon Dioxide or Sucrose*

Results from these experiments are illustrated by autoradiographs (Plate 1). The images here are faint, but the original negatives show good resolution. The close correspondence between photographs of sections and their autoradiographs permits results to be presented in the latter form. Sections of kernels from untreated plants did not produce any reaction on the photographic plates.

(i) $^{14}CO_2$.—Prior variety barley plants grown in potting compost were used. Ears were treated on one of the following occasions: at 3-day intervals to 18 days, then at 27, 36, and 45 days after anthesis. After treatment the ear was removed from the assimilation apparatus and the intact plant transferred to the open. Two kernels were taken as samples at 1, 3, 9, 15, 21, 30, and 39 days after treatment, freed of glumes, and stored in absolute ethanol. The distribution of carbon-14 within the kernel is illustrated in selected autoradiographs (Plate 1, Figs. 1 and 2).

When $^{14}CO_2$ was supplied at 3, 6, and 9 days, radioactivity became concentrated in the peripheral tissues, especially in the furrow of the kernel. As the kernels matured radioactivity persisted in these tissues, i.e. the pericarp. Traces of radioactivity were detected 1 day after feeding in the small endosperm of kernels treated at 3 days, and traces were still present at maturity. Feeding between 12 and 18 days led to a greater accumulation of carbon-14 in the endosperm than the pericarp, and distribution was uniform throughout the former tissue. At 27 days the response was similar although, at this stage and increasingly through to 45 days, relatively less radioactivity was evident in the centre of the endosperm. However, the lowered activity of this zone was not as marked as in the corresponding tissue supplied with ^{14}C-sucrose.

Table 1

MEAN DRY WEIGHT PER KERNEL (MG) FROM SHADED AND UNSHADED TREATMENTS FOR THE THREE BARLEY VARIETIES (15 REPLICATES)

Variety	Treatment		Difference	Level of Significance (%)
	Unshaded	Shaded		
Awnless	27·9	25·4	2·5	10
Single-awned	23·6	18·0	5·6	1
Triple-awned	42·7	34·8	7·9	0·1

(ii) ^{14}C*-sucrose*.—Ears were taken for sucrose feeding at the same times after anthesis as for $^{14}CO_2$ feeding. Two kernel samples were taken immediately after the radioactive sucrose was replaced with water, then at 1, 2, 4, 6, and 9 days. After removal of the glumes, kernels were stored in absolute ethanol. The distribution of carbon-14 within the kernel is illustrated in Plate 1, Figures 3 and 4. Between anthesis and 9 days radioactivity was concentrated towards the periphery in the regions corresponding to the pericarp. From 12–27 days concentration was mainly in the endosperm and uniformly spread while from 36–45 days greatest concentration had again reverted to the periphery. The amount of carbon-14 supplied as sucrose that was fixed in ethanol-insoluble forms reached a maximum within 24 hr of feeding, although little fixation occurred during the actual feeding, as shown by the "zero" samples. This result allows of two interpretations: accumulation of sucrose into the endosperm may be rapid, but its conversion into insoluble compounds slow;

accumulation of sucrose into the stem and rachis may be rapid, but its entry into the kernel slow.

Soon after feeding either $^{14}CO_2$ or ^{14}C-sucrose the distribution of radioactivity was very similar in both cases; the subsequent differences are attributed to the continued growth of kernels on the ears of intact plants in contrast to the cessation of growth in excised ears. In this connexion where $^{14}CO_2$ was fed at 18 days, or earlier, carbon-14 was present in highest concentrations no later than 1 day after treatment, and thereafter the concentration of radioactivity, as judged from the autoradiographs of whole kernels, decreased. No decrease was observed following ^{14}C-sucrose feeding. The decrease in the first instance could be due to loss by respiration or translocation of resolubilized materials, or dilution in the expanding cells or to all three.

(iii) *Distribution of Carbon*-14 *between Different Chemical Fractions*.—The fractions examined were: those soluble in 80 per cent. ethanol—mainly sugars; those insoluble in ethanol but soluble in 33 per cent. chloral hydrate—mainly starch; those insoluble in either of these solvents. Radioactivity was detected in all three fractions from kernels of ears supplied $^{14}CO_2$ 18 days after anthesis. No change in either "starch" or "residue" components was apparent between 1 and 27 days after treatment. This supports a previous notion that the decrease in concentration of radioactivity noted in autoradiographs of kernels of ears subsequent to feeding is due to dilution by non-radioactive materials. However, radioactivity in the ethanol-soluble fraction decreased with time and at maturity was negligible. The fate of this carbon-14 remains uncertain; losses by translocation are unlikely (see Sections III (*d*) and III (*e*)), but it could have been respired or transformed into insoluble forms. The present evidence allows no choice between the alternatives; nevertheless, these losses are small compared with the radioactivity permanently retained within the starch and residue components.

(*c*) *Competition between Kernels for Substrate from Outside the Ear*

Prior variety barley was sown at 10 seeds per pot in sand culture and later thinned to six per pot. The following six treatments were represented in every pot by assigning, at random, each treatment to one of the six plants: (1) 18 florets per head; (2) 12 florets per head; (3) 6 florets per head; (4) 6 florets per head plus 12 empty florets; (5) 18 florets per head, stems defoliated and shaded; (6) 6 florets per head, stems defoliated and shaded. All ears on any one plant were treated. There were harvests at 15, 25, 35, and 45 days after anthesis, three pots being used for each harvest. Only six kernels (corresponding ones in all cases) were taken from each head at harvest, the glumes were removed, and the kernels of each plant combined and oven dried.

The progressive change of kernel weight when ears carried different numbers of kernels (treatments (1), (2), and (3) above) is illustrated in Figure 2. The three values were almost identical at harvests up to 25 days, thereafter variability increased although differences were not significant at the 5 per cent. level. The average final weight of kernels from these treatments was 44·5 mg per kernel. It is concluded that in plants grown under these conditions there is no competition between kernels for substrates.

The situation appears very different when the additional treatments of stem defoliation and shading are applied. The weight of kernels from ears bearing 18 florets was significantly less than corresponding unshaded ones at all harvests ($P<0\cdot05$). That from ears bearing six florets was also less than corresponding unshaded ones at 15, 25, and 45 days ($P<0\cdot05$) although at 35 days the difference did not quite attain the 5 per cent. level of significance. Furthermore, differences between these last two treatments themselves were highly significant at 25, 35, and 45 days; at maturity kernels from the 18-kernel ear weighed 19·9 mg, those from the six-kernel ear were 31·8 mg. Therefore, it is further concluded that where substrate supply is greatly curtailed competition between kernels arises, and moreover, the competition is evident throughout growth.

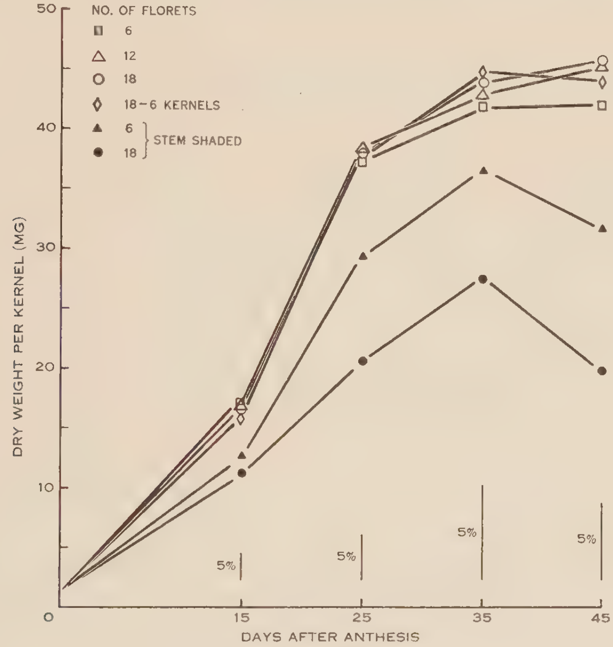


Fig. 2.—Dry weights per kernel during development from ears carrying different numbers of kernels, and with stems unshaded and shaded. Least significant differences at 5 per cent. level also shown.

(d) *Translocation between Kernels of Substrate Assimilated within the Ear*

Ears of Prior variety were used 24 days after anthesis, when either the top or bottom portions were supplied $^{14}CO_2$ for 4 hr. After treatment, ears were removed from the assimilation apparatus and the whole plant allowed to mature in the open. The bottom portion of one ear, whose top portion was fed $^{14}CO_2$, was subsequently de-awned and shaded. At maturity two kernels (the apical and basal) were taken from both portions of treated ears and autoradiographs were made of their transverse sections. The findings are set out in Table 2.

Thus there seems little doubt that the carbon-14 assimilated by the upper half of the ear was not translocated to kernels in the lower half, even when these kernels were shaded and hence entirely dependent on substrate from elsewhere for their subsequent maturation. However, there is some doubt as to whether carbon-14 assimilated by the lower half of the ear (treatment (3)) was translocated upwards, despite the fact that traces of radioactivity were recorded. This result may have been due to dark fixation of $^{14}CO_2$ *in situ*, since, unlike the other treatments, both halves of the ear were here exposed to $^{14}CO_2$ (see Section III (*e*)(ii)). The rachii and awns showed responses similar to those of the kernels. Awns from the top half of the ear (treatment (3)) appeared to accumulate concentrations of radioactivity comparable to the kernels. This supports the notion that carbon-14 arises in this instance from dark fixation, and not translocation, otherwise kernels, which act as sinks for substrates, should accumulate more than awns.

Table 2

TRANSLOCATION BETWEEN KERNELS OF SUBSTRATE ASSIMILATED WITHIN EARS OF PRIOR VARIETY BARLEY

Treatment No.	Treatment of Ears	Location of Kernels	Radioactivity
I	Top half fed $^{14}CO_2$	Top half Bottom half	Present Absent
II	Top half fed $^{14}CO_2$; bottom half subsequently de-awned and shaded	Top half Bottom half	Present Absent
III	Bottom half fed $^{14}CO_2$	Top half Bottom half	Traces Present

(*e*) *Translocation of Substrate Assimilated within the Ear to other Sites*

Autoradiographs were made of the flag leaf, the flag leaf sheath, and portions of the stem from shoots whose ears were supplied with $^{14}CO_2$ at 3, 15, or 27 days after anthesis. These revealed that carbon-14, assimilated by the ear at 3 days, was subsequently distributed throughout the internode between the flag leaf and the ear. However, there was no trace of activity in the stem below this node, in the flag leaf sheath, or in the flag leaf. No trace of radioactivity was found outside the ear when $^{14}CO_2$ was supplied at either 15 or 27 days after anthesis.

IV. Discussion

Application of the ear-shading technique to awnless, single-awned, and triple-awned varieties of barley led to reductions in yield per kernel of 9, 23, and 18 per cent., respectively. The contribution of photosynthesis in the ear itself to grain yield may depend on the assimilatory activity of the ear. If so, these activities should approach the proportions by which ear shading reduced yield, i.e. 3 : 8 : 6. Previous estimates have given the relative assimilatory activities of ears of these three varieties as 3 : 7 : 10 (Buttrose 1956). Attention has already been drawn to the fact that the reductions in yield by shading were not significantly different from each other, and since the two relationships are so close it is difficult to decide whether there is a real difference between them or not. Results presented here throw no further light on this matter, but lend some support to the proposition (see below) that discrepancies between shading estimates arise because of compensatory mechanisms in the plant. Thus, under some circumstances or with some varieties certain sources of carbon substrate may replace others rendered ineffectual (e.g. by shading). It is to be noted that in such circumstances the two above relationships need not be the same to conform with the proposition that ear shading reduces yield only by preventing photosynthesis.

Despite the uncertainties in interpreting shading experiments, it has been concluded that carbon dioxide fixed in the ear makes a substantial contribution to yield. This work, using carbon-14 to trace ear assimilate, lends strong support to this conclusion. Radioactivity supplied as $^{14}CO_2$ to the ear was permanently incorporated into kernels throughout development. It seems equally clear that carbon substrate translocated from other organs also makes a major contribution, since ^{14}C-sucrose supplied through the stem was always as readily, and as permanently, incorporated into the kernel. Whatever the source, the starch and other alcohol-insoluble carbon fractions remained apparently unchanged between 24 hr after carbon-14 was supplied and maturity. Again, the source was without influence on the site of incorporation within the kernel. However, the stage of kernel development influenced the site: early, accumulation was greatest at the periphery of the kernel; throughout the greater part of development, accumulation was greatest in the endosperm and uniformly distributed therein; near maturity, accumulation was again greatest at the periphery. These changes probably reflect alterations in synthetic activities during kernel development.

When carbohydrate supply was greatly reduced by defoliation and stem shading, competition between kernels occurred for storage products of the stem. Under normal conditions competition was not observed, hence substrate was not limiting. It is admitted that in this experiment the maximum kernel number was 18 per ear, whereas a normal ear carried about 23 kernels. However, it was found that final weight per kernel (where there were 18 per ear) was not significantly different from that of kernels where there were 28 per ear (unpublished data). It is of interest that Archbold (1945) has also suggested that there is an excess of carbohydrates in the barley plant, sugar accumulating as an inevitable consequence of assimilatory products exceeding growth and respiratory requirements.

The above results confirm the now commonly held view that several different sources in the plant contribute to kernel development. As a speculation it is now proposed that these several sources comprise a general pool from which the kernel derives its substrate, and moreover, a deficiency in one source may be offset by an added contribution from another. A tentative scheme which accommodates these suggestions is presented in Figure 3. If there is a general surplus of carbohydrates (scheme B) the ear could contribute, in the normal plant, as much as 50 per cent. of its kernels' dry weight, but ear shading might assess this contribution at only 33 per cent. because of induced contributions from other sources. If there is not a surplus of carbohydrates (scheme A) shading techniques might give an accurate assessment. There is, however, no *a priori* reason for assuming that the conditions of scheme A always apply; in other words, that all potential sources always make their maximum contribution or that their contribution is constant. Notwithstanding these considerations the ear is represented (scheme B) as contributing its maximum to kernel development. This conclusion follows the findings that exposure of ears to $^{14}CO_2$ between 15 and 27 days after anthesis (the period of rapid kernel development) gave no evidence of translocation to organs outside the ear, and that only in the early stages of kernel development was some ear assimilate translocated down the uppermost internode. Furthermore, the evidence suggests that even translocation between kernels within an ear did not occur.

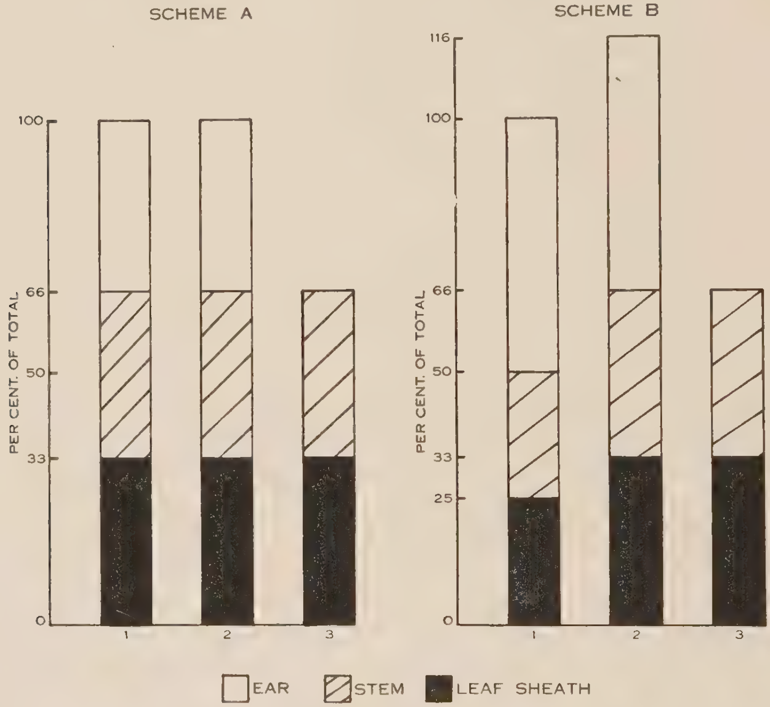


Fig. 3.—Two schemes showing: *1*, actual contributions by plant parts to kernel development; *2*, potential contributions; *3*, contributions assessed by ear shading. Scheme *A*, classical interpretation—plant parts always contribute to a maximum; scheme *B*, proposed interpretation—some parts not always contributing to their maximum potential.

Where there is competition for substrate between kernels an assessment of the maximum contribution of photosynthetic products of the ear to kernel yield can be made. Thus in the competition experiment described it may be assumed that the contribution of each floret remains constant. If each floret contributes x to its kernel weight then the total contribution from the ear to kernel yield per head will be nx, where n is the number of kernels per head. When the final weight per kernel is w the yield per head is nw, and the non-ear contribution $n(w-x)$. In competitive conditions it may now be assumed that the same amount of material enters the ear from other plant parts, irrespective of the number of kernels per head. Thus the following equation,

$$n_1(w_1-x) = n_2(w_2-x),$$

can be derived for two ears showing competition for substrate, bearing n_1 and n_2 kernels, and each kernel weighing w_1 and w_2 respectively. A solution for x allows the calculation of percentage contribution by ears in the normal plant as $x/w_0 \times 100$, where w_0 is the weight of kernels which develop on control plants.

The application of this method to the inter-kernel competition experiment, where defoliation and stem shading were imposed on plants with either six or 18 kernels per head, gives the following results:

No. of Days	Ear Contribution (%)
15	63
25	43
35	64
45	31

No claim is made that these results represent accurate estimates; the experiment was designed to study inter-kernel competition. But the approach has much to commend it. It is a maximum contribution that is measured rather than a minimum. Furthermore, the estimate (x) is obtained from two treatments identical with respect to shading and hence it is independent of secondary effects of shading. Its further application may contribute to a better understanding of the problem—how much carbon substrate for kernel development arises from the several potential sources of the normal plant?

V. Acknowledgment

The barley varieties used in this work were supplied by Dr. K. W. Finlay, Plant Breeding Section, Waite Agricultural Research Institute. The authors wish to express their gratitude for his assistance.

VI. References

Appleman, C. O., and Eaton, S. B. (1921).—*J. Agric. Res.* **20**: 795.

Archbold, H. K. (1942).—*Ann. Bot., Lond.* (N.S.) **6**: 487.

Archbold, H. K. (1945).—*Nature* **156**: 70.

Archbold, H. K., and Datta, C. (1944).—*Ann. Bot., Lond.* (N.S.) **8**: 363.

Asana, R. D., and Mani, V. S. (1950).—*Physiol. Plant.* **3**: 22.

Asana, R. D., and Mani, V. S. (1955).—*Physiol. Plant.* **8**: 8.

Buttrose, M. S. (1956).—Honours Thesis, University of Adelaide.

DONALD, C. M. (1954).—*Aust. J. Agric. Res.* **5**: 585.

FUJIWARA, A., and SUZUKI, M. (1957).—*Tohoku J. Agric. Res.* **8**: 89.

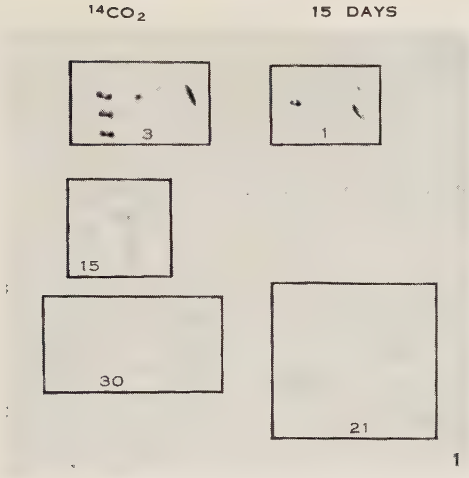
KRAVCOVA, B. E. (1957).—*Dokl. Akad. Nauk S.S.S.R.* **113**: 1163. (*Field Crop Abstr.* **11**: 41 (1958).)

PORTER, H. K., PAL, N., and MARTIN, R. V. (1950).—*Ann. Bot., Lond.* (N.S.) **14**: 55.

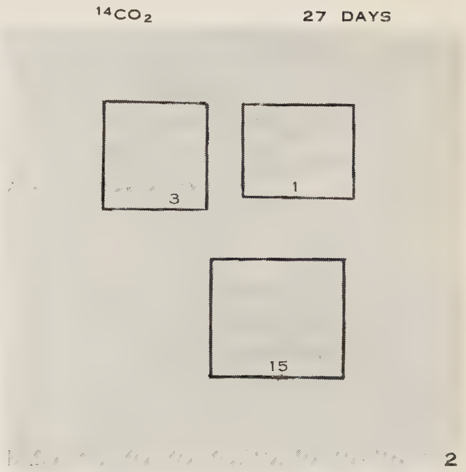
VERVELDE, G. J. (1953).—*Netherl. J. Agric. Sci.* **1**: 2.

WATSON, D. J., and NORMAN, A. G. (1939).—*J. Agric. Sci.* **29**: 321.

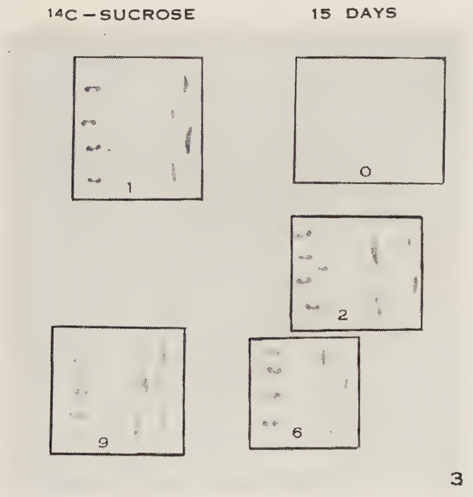
PHYSIOLOGY OF CEREAL GRAIN. I



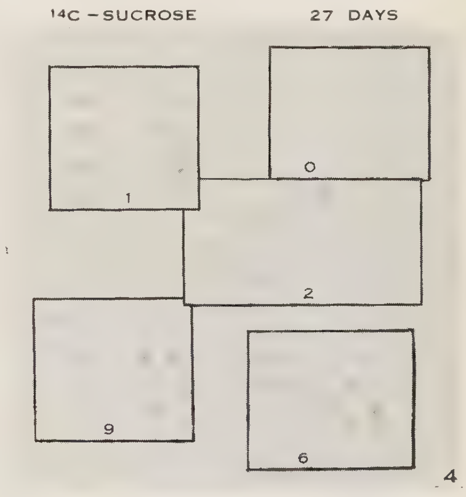
1



2



3



4

Autoradiographs of sections of kernels

Figs. 1 and 2.—Autoradiographs from heads treated with $^{14}CO_2$ at 15 and 27 days after anthesis. Days after treatment when samples taken indicated for each group.

Figs. 3 and 4.—Autoradiographs from heads treated with ^{14}C-sucrose at 15 and 27 days after anthesis. Days after treatment when samples taken indicated for each group.

FORMATION OF THE COLONY IN THE FUNGUS *CHAETOMIUM**

By N. J. B. Plomley†

[*Manuscript received May* 5, 1958]

Summary

Growth in the fungus *Chaetomium globosum*, on agar medium, is decribed in its following aspects: growth of the hypha, increase in size of the colony, and change of density of the hyphae within the colony.

Individual hyphae increase in length at constant rate but branching produces exponential growth in a mass of hyphae.

The colony has been studied from germination of the spore until a constant rate of marginal expansion is set up. On germination there is a lag phase of growth; this is followed by acceleration of growth to an exponential phase; and finally a steady state is set up in the margin so that it expands at constant rate.

It is concluded that the formation of the colony can be described on the basis of growth of the hyphae, by postulating a *growth unit*. This is defined as a free-growing hyphal tip associated with a growing mass of constant size in constant environment.

I. Introduction

Although fungal colonies have been the subject of innumerable physiological studies, the way in which growth proceeds in the colony and the question whether growth concerns the mass of mycelium as a whole or becomes restricted to some part of it have hardly received attention.

It is the purpose of this paper to describe how growth proceeds in a fungal colony grown on a solid medium. This involves descriptions of growth in the colony and in the individual hyphae, and the correlation of these to describe the formation of the colony.

The fungus studied was the ascomycete *Chaetomium globosum*.

II. Experimental Methods

(*a*) *Culture Medium*

The standard medium upon which the fungus was grown was 2 per cent. malt agar. When the effect of starvation was studied, plain agar was used as the culture medium. In the general experiments, the medium was contained in petri dishes 10 cm in diameter which had been selected for uniformity, particularly for an even thickness of glass in the bottom. The dishes were filled with medium to a depth of 6–8 mm. The colonies were grown at a constant temperature of 28°C.

---

*The work was carried out partly at the University of Tasmania and partly at the University of Sydney.

†Department of Anatomy, University of Sydney.

(*b*) *Methods of Culture*

Except for an experiment in which hyphal-tip inoculation was used, all colonies were grown from single spores. Such inoculations were made by picking up a single spore from a group of spores on agar, using a fine platinum point and high-power binocular magnification, and transferring it to a marked site on an agar plate. Such inoculations were inspected to ensure that only single spores had been transferred.

Hyphal tips were inoculated by transferring a hyphal tip from the margin of a colony by the above method.

(*c*) *Measurements*

(i) *Rate of Increase in Length of Individual Hyphae.*—This was studied by photographing hyphae from time to time. Enlargements were made of these photographs, from which hyphal lengths were determined.

(ii) *Rate of Enlargement of Surface of Colony.*—Each colony was measured along two axes at right angles and an average of the measurements used. This method gave rise to some error in very young colonies which, consisting of only a few hyphae, were usually not circular.

Colonies having a radius less than 1–2 mm were measured under the microscope, using ocular micrometers. For measurements up to about 100 μ, the micrometer unit was equal to about 17·5 μ and readings could be estimated to about one-third of a unit, i.e. to about 6 μ; in the range 100–1000 μ the micrometer unit was 87·5 μ and readings could be estimated to about one-quarter of a unit, i.e. to about 22 μ.

(iii) *Rate of Increase in Length of Mycelium after Germination.*—This was studied during the first few hours of growth after germination of the spore, from a photographic record. Length of mycelium was determined from suitable enlargements. Phase-contrast microscopy was used.

(iv) *Effect of Depth of Medium on Surface Growth.*—To determine whether depth of medium affected rate of growth of the surface mycelium, a technique was required which would minimize all variables other than depth of medium. For example, it was found that the rates of radial enlargement of colonies grown on shallow (1–2 mm) and deep (8–10 mm) media were usually, though not always, faster on the shallow medium in young colonies, but faster on the deep medium in older colonies (5–30 mm radius). An experiment was therefore set up in which the one plate was divided into shallow (0·5 mm) and deep (4·5 mm) regions by placing a thick slab of glass in it. Spores were then plated on the surface of the medium along the line of the edge of the slab.

(v) *Growth of Colony below Surface of Medium.*—Growth of mycelium into the medium was measured in vertical sections through the colony. The scales used were the same as for measurements of surface growth. A series of measurements was made on colonies grown in petri dishes.

In another series the effect was studied of restriction of the surface of the medium to a small area. In these experiments colonies were grown in tubes of 7 mm and 12 mm radius, and the depths to which hyphae grew were measured in vertical sections through the colony. In long experiments there was shrinkage in the agar column: this was allowed for in determining the depth of growth.

(vi) *Density of Hyphae over Surface of Colony.*—Regional densities of surface hyphae were determined from photographs of the surfaces of colonies. Tracings of the surface hyphae were made from these photographs—being in sharpest focus they could usually be distinguished from other hyphae. On the tracings concentric rings were described about the centre of the colony. Hyphal lengths were measured in each ring and unit densities calculated; in some of the larger colonies the measurements were made on a sector or sectors, or upon other areas, when the whole colony could not be dealt with satisfactorily. From such measurements also the whole length of hyphae in the surface of colonies could be determined.

Table 1

LENGTHS (μ) OF THREE HYPHAE* AT SUCCESSIVE TIMES OF MEASUREMENT

Hypha No.	Part Measured	Time of Measurement (hr min): 0.00	1.15	2.50	4.30	6.30	9.50	12.40
I	Main hypha	44	67	115	168	235	342	437
	Branch hyphae	—	—	—	10	24	288	778
	All hyphae	44	67	115	178	259	630	1215
II	Main hypha	72	119	147	187	226	336	
	Branch hyphae	—	—	19	45	79	403	
	All hyphae	72	119	166	232	305	739	
III	Main hypha	62	101	154	211	269	349	
	Branch hyphae	—	—	—	61	255	837	
	All hyphae	62	101	154	272	524	1186	

*The correlation coefficients for the three sets of data (omitting the first point of each (see second footnote, p. 56)) are all highly significant—arithmetic scale: I, 0·998; II, 0·990; III, 0·997; logarithmic scale: I, 0·996; II, 0·927; III, 0·998.

III. Morphology

The spores of *Chaetomium globosum* were thick-walled and ellipsoidal, the axes measuring about 8 and 9 μ. Germination occurred 2–2½ hr after plating, the germ tube being protruded from a pore at one end of the spore.

The young colony consisted of one, perhaps two, hyphae, usually unbranched at first, so that their elongation produced asymmetry. As branching proceeded, more and more of the free space around the spore became occupied, so that the colony gradually became circular, this form being attained fully in a colony of about 1 mm radius. As the colony enlarged further, the arrangement of the marginal hyphae became stabilized, the hyphae mostly pointing radially outwards in the advancing border. Such a stabilized arrangement of marginal hyphae was associated with a constant rate of marginal expansion.

The mycelium was septate, the "cells" being multinucleate and their length rather variable (13–48 μ). Septa were not found close to the tips of the hyphae, so that there was a long apical segment (46–120 μ). The diameters (2·8–4·2 μ) of hyphae formed in the margin of the colony were greater than the diameters (0·8–2·2 μ) of hyphae formed subsequently within the colony; the latter were progressively finer as branching continued. Hyphal diameters were smaller in starved colonies.

IV. GROWTH IN THE COLONY

(*a*) *Growth of a Hypha*

The rate of growth of individual hyphae was studied by measuring the increase in length of hyphae and their branches. Measurements on three hyphae* are given in Table 1 and those on hypha I are plotted on arithmetic and logarithmic scales in Figure 1. They show that:

(i) Growth in length of an individual hypha proceeds at constant rate (Fig. 1, *EF*, (*AD*)).

(ii) Growth in a hypha and its branches is exponential,† the amount of new hyphal material added being proportional to the mass present (Fig. 1, *BC*).

Linear growth of individual hyphae is associated with a continual increase in the number of hyphae, so that growth in the whole mass is proportional to the amount present and the growth curve is exponential. Branching occurs at *A* (Fig. 1) and this represents a length of hypha of the order of 130 μ (region of log 2·10 μ). In six other sets of measurements the point *A* varied between 110 and 180 μ (average of seven measurements 150 μ).

(*b*) *Increase in Size of Colony*

Rate of growth of the colony was measured in the first place as rate of expansion of the mycelium over the surface of the medium. The relationship between growth over the surface and growth into the medium was then investigated. The effect of depth of medium upon growth of the colony was studied (1) when the area of the surface of the medium was unlimited but its depth was restricted, and (2) when the area of the surface was restricted but its depth was unlimited.

Rate of change of mean colony radius (Fig. 2) was exponential until the radius was about 0·1 mm. This phase was followed by a period of declining logarithmic rate but increasing linear rate until, at a radius of 3–5 mm, the rate of radial expansion became constant. Thereafter, it continued at constant rate until obstructed, the largest colonies measured still enlarging in this way at a radius of about 70 mm.

---

*These measurements were made on hyphae growing in a hyphal-tip inoculation; similar results were obtained for hyphae in young, single-spore colonies. The hyphae measured, and their branches, were of the same diameter.

†The part *BA* of the graph *BC* (Fig. 1) deviates from the straight line because the whole of the growing mass is not being included in the measurements. This can be shown by adding an equal amount to all values, making the first value about 150 μ, when the initial deviation disappears. Over-compensation produces an opposite deviation.

Starvation had the effect of reducing the rate of growth. The reduction in the exponential rate was not apparent when measured in terms of colony radius, but was shown by measurements of the total length of hyphae in the surface.

The measurements of colony radius plotted in Figure 2 do not give any information about the earliest growth of the colony, during the first few hours after germination. Rate of increase in length of the mycelium during this period was linear, with equal increments of length in unit time (Fig. 3). This constant rate does not appear to continue for long and there is some evidence that the rate is accelerating in mycelium 50 μ long.

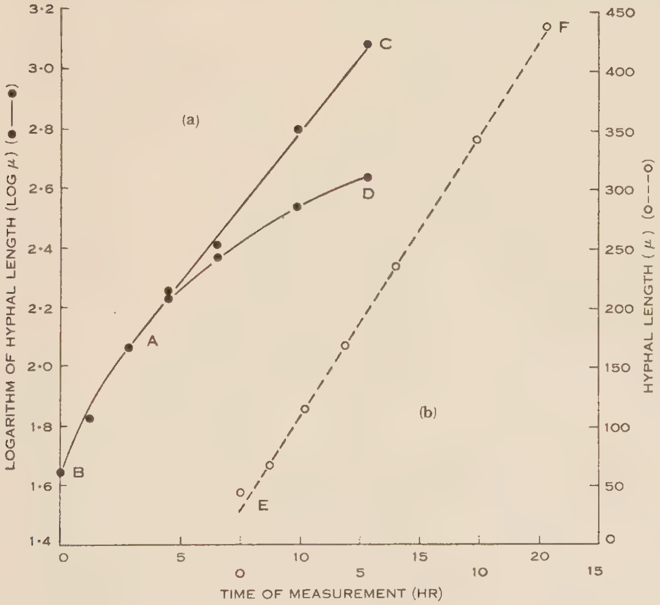


Fig. I.—Growth of hypha I. (*a*) Logarithm of hyphal length (log μ) plotted against time of measurement: *BA*, unbranched main hypha; *AC*, main hypha with its branches; *AD*, main hypha alone. (*b*) Rate of elongation of main hypha alone.

Growth downwards into the agar medium kept pace with growth over its surface, making the colony hemispherical. The least squares equation for data on a series of colonies in size between 0·1 and 8·5 mm radius was

$$y = 1{\cdot}0032\,x + 0{\cdot}048,$$

which is very close to the straight line $y = x$ passing through the origin.

Growth over the surface of the medium was not affected by the depth of the medium—the surfaces of colonies in plates in which the medium was partly deep and partly shallow were circular.

When colonies were grown in tubes, restricting the surface of the medium, growth downwards kept pace with that over the surface for a time, but slowed markedly when the surface hyphae reached the wall of the tube. It is suggested tentatively that this slowing was associated with inefficient gaseous diffusion, due to occlusion of the surface.

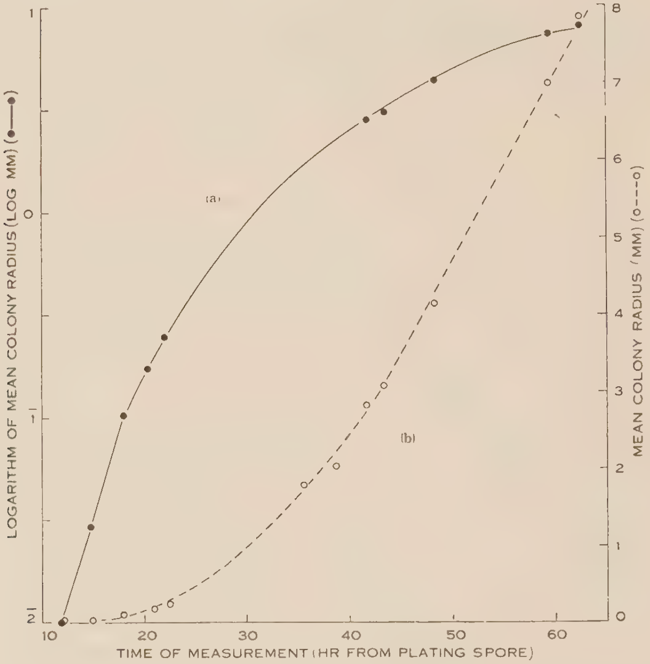


Fig. 2.—Increase in size of surface of colony. (*a*) Logarithm of mean colony radius plotted against time of measurement. (*b*) Rate of increase of mean colony radius.

(*c*) *Density of Hyphae within the Colony*

The distribution of hyphae in a colony was studied by measuring change of hyphal density over its surface. Hyphal lengths were measured in concentric rings of surface and unit densities calculated by the formula:

$$d_1 = l_1/(r_1^2 - r_0^2),$$

where d_1 = density over the ring,

l_1 = length of hyphae in ring,

r_1 = outer radius of ring, and

r_0 = inner radius of ring.

The curves of density variation over the surface of the colony are of two types (Fig. 4). In colony (*a*) (radius <0·216 mm) change in density is exponential from the centre outwards, but in larger colonies ((*b*)-(*g*)) change in density is exponential only in the margin and inside this changes more and more slowly.

The curves also bring out three other features of growth in the colony. Firstly, the decline from the marginal exponential phase began at about the same density in all the colonies, at a value of 10–20 units (1·0–1·3 on log scale). In other words,

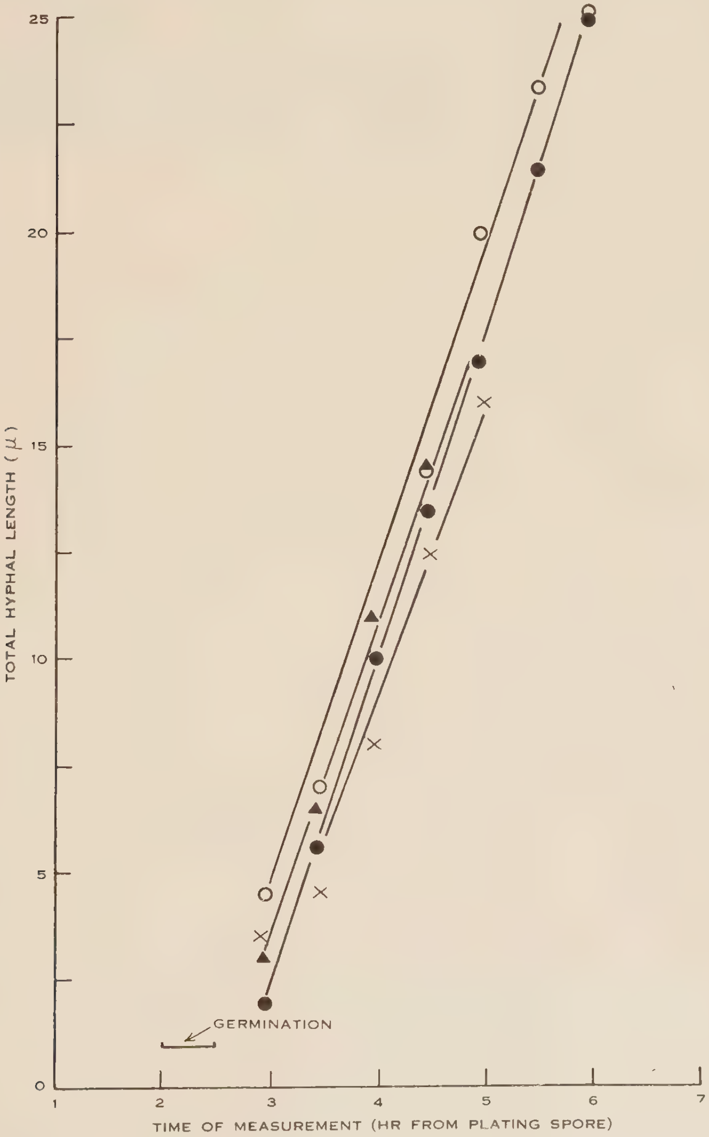


Fig. 3.—Rate of increase in length of mycelium in first few hours after germination. Individual measurements for four colonies (●, ○, ▲, and ×).

under the conditions of experiment the medium supported exponential growth only so long as the density of hyphae did not exceed 10–20 units. This is also shown in Figure 5 in which change of density is followed in some region, growth rate there increasing exponentially at first but declining after a time. Secondly, density tended towards a maximum value, hyphal saturation extending outwards progressively as the colony enlarged. Thirdly, the extent of the outer fringe of exponential growth was of the order of 0·1 mm (100 μ).

The decline in growth rate within the central mass is actually more pronounced than the density determinations show. The latter are based on measurements of the *length* of hyphae and take no account of their diameter: hyphae formed within the colony are progressively finer as branching proceeds.

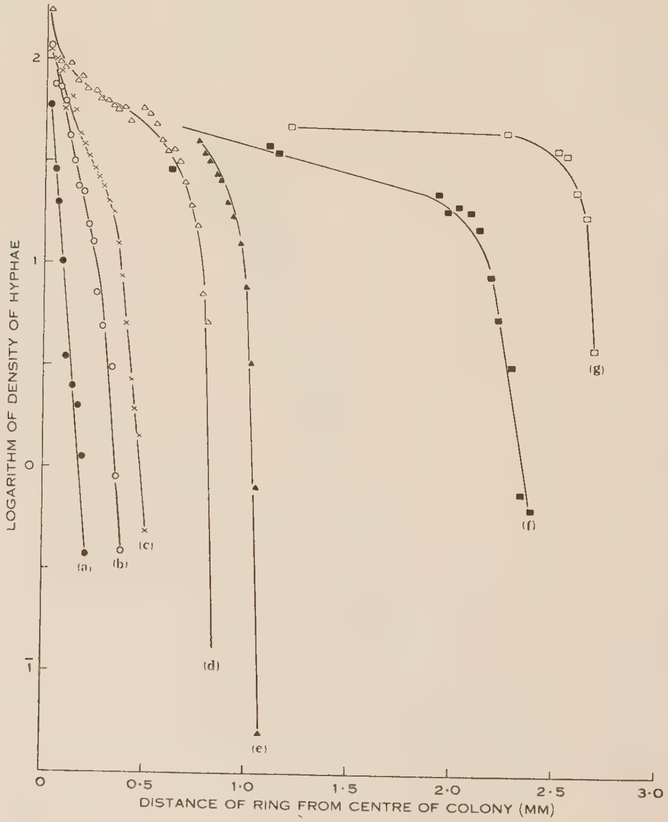


Fig. 4.—Variation in hyphal density over the surface of colonies of various sizes. Logarithmic scale.

In the larger colonies regional counts showed that branching followed a similar pattern to hyphal density. The counts did not permit accurate determination of this feature, but the difference between the marginal and central regions was clear.

Change of density in colonies grown on plain agar, and therefore starved, showed the same features as in colonies grown on the standard medium, but the density values were less.

V. Discussion

The picture built up by the measurements and observations reported above shows that growth in *Chaetomium* has its basis in the characteristics of hyphal growth: hyphae increase in length at constant rate, but because they branch from time to time, increase in length of the branching mass of hyphae is exponential.

Before considering the mechanics of growth in *Chaetomium* further, relevant published work will be reviewed briefly. Only three papers, one by Castle (1940) and two by Smith (1923, 1924), have been seen which deal with the manner of growth of fungal hyphae. Castle studied the growth of the sporangiophore of *Phycomyces*, a cell attached at one end to the mycelium from which it is nourished. More relevant to the present work, however, are Smith's studies of the growth of hyphae in several species of fungi.

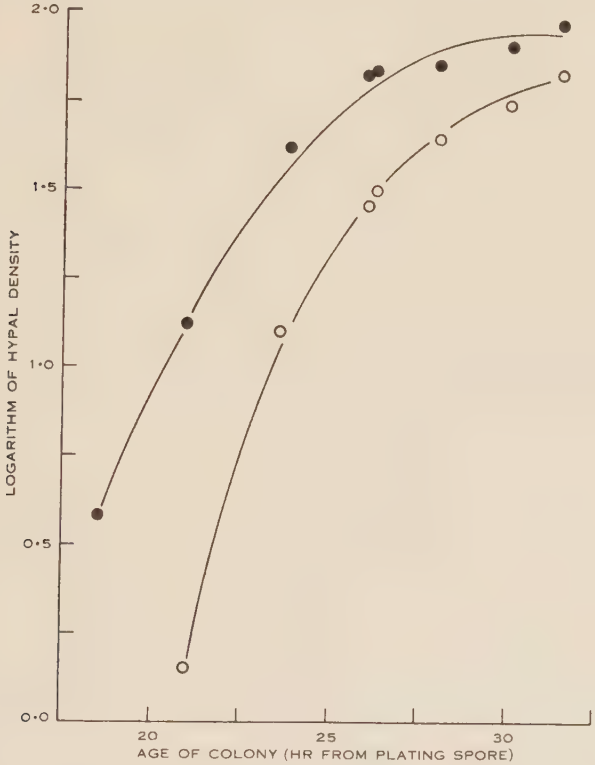


Fig. 5.—Variation in hyphal density at sites 0·12 mm (●) and 0·24 mm (○) from the centre of colonies of various sizes. Logarithmic scale.

Smith (1923, 1924) found in representative fungi of widely separated genera that growth in length of a hypha was purely apical, and observed that cross walls were not laid down in the tip of the hypha, i.e. the length of the apical segment was greater than that of segments behind it, usually in the proportion of about 3 : 1. In the present work on *Chaetomium*, also, growth of the hypha was apical, and the apical segment was longer than those behind it, again in the proportion of about 3 : 1.

Smith (1924) showed that in *Botrytis* growth of a hypha with its branches was proportional to the total length of hyphae present, but that the growth rate of an individual hypha declined from the exponential rate—his measurements show that the rate of growth of the individual hypha was linear. Measurements given in his earlier paper (1923) show that growth of the hypha in *Fusarium* and *Pyronema* was linear also.

Smith (1924) also drew attention to differences in rate of growth between different hyphae and in the same hypha at different times. Similar observations were made on *Chaetomium* in the present work: the differences appear to be normal variation.

Leaving aside for the present any consideration of whether the growth rate of hyphae is intrinsically linear or intrinsically exponential, the formation of the colony in *Chaetomium* can be described on the basis of growth of the hyphae.

On germination of the spore, the mycelium, usually a single unbranched hypha, grows at constant rate for a time (Fig. 3), but later the growth rate increases until the colony is growing exponentially. Such exponential growth involves not only the individual hyphae, as shown by exponential increase in the radius of the colony (Fig. 2), but also the whole mass of mycelium, as shown by the exponential change in density of the hyphae (Fig. 4, colony (*a*)).

Thereafter, growth in the colony can be considered from two points of view, growth in the margin and growth within the colony. Growth takes place in the margin in such a way that the radius of the colony increases at constant rate (Fig. 2). Within the colony growth not only slows down progressively (Figs. 4 and 5), but the hyphae formed there are of smaller diameter, evidently a response to less favourable conditions for growth (*vide* starved growth), an instance of biological adaptation. A maximum hyphal density is attained gradually within the colony, there being a gradient between a central mass of maximum density and the margin (Fig. 4).

While the above remarks upon growth in the colony refer more particularly to growth in its surface, they are also generally applicable to the colony as a whole. The colony grown from a spore plated on the surface of the agar has the form of a hemisphere, growth proceeding radially into the medium at the same rate as growth in the surface.

The characteristics of hyphal growth appear to point to a functional unit of growth in *Chaetomium* rather than a morphological one; this will be referred to as a *growth unit*. This notion is based principally upon the observations on the growth of individual hyphae (Fig. 1), the hypha increasing in length at constant rate (linear growth), while the increase in length of the mass of hyphae is proportional to its amount (exponential growth) and is associated with branching. Branching occurred when the hypha was of the order of 150 μ long (at A, Fig. 1), which is somewhat longer than the apical segment.*

Considered functionally, the growth unit will be a free-growing hyphal tip associated with a growing mass of constant size in constant environment. This is possible because of the tubular form of the hypha, the growing tip as it were continually leaving behind itself material in which growth is independent. In the growth unit, growth might be intrinsically exponential, i.e. proportional to the amount of growing material; or it might take place at constant rate, the occurrence of branching as the hypha becomes a certain length producing exponential growth in the mass of hyphae. Where growth involves the elongation of a tube there is little real difference between these two points of view. This tubular form also frees the hypha from the restriction of a limiting surface/volume ratio, and rate of growth is then subject to modification only by the environment.

It is, perhaps, easier to visualize growth in *Chaetomium* as being essentially exponential, and in these terms constant radial expansion of the margin will result from branching, and its rate will be the *value* of the exponential when the growth unit is at its full length (150 μ), this length depending upon the environment and upon the genotype. The initial linear phase on germination will represent a true lag phase of growth, to be explained in terms of some limiting factor in growth and metabolism, or of the necessary organization of the materials of the spore for growth (cf. Hinshelwood 1946). Recent work by Machlis (1957), showing that certain substances (e.g. glutamic acid) will reduce the lag phase, is relevant to this, although the "lag phase" of his studies is a composite period including all changes up to the rounding-off of the colony.

The principal difficulty in visualizing growth as being linear lies in there being a marked difference between the linear rate in the hyphae of the lag phase and the linear rate in the marginal fringe, the former being only about one-third of the latter. However, limiting factors can be brought forward in argument here.

Lastly, brief comment may be made upon the phenomenon of branching, the process which leads to the occupation of the medium. Presumably, branching occurs because the hypha cannot maintain exponential growth indefinitely—because extension of the mass is localized in the growing tip. Such localization will subject increase in length of the hypha to the limitation of the deposition of new wall material there, itself dependent upon many factors, including translocation of materials within the hypha. Such limitation will impose a constant rate of elongation upon the hypha, but the mass of hyphae can continue to grow exponentially because branching occurs. In fact a steady state is set up in the margin and this is expressed in a constant rate of marginal expansion. The independence of the growth units in the mass is seen clearly when the space available for growth is varied. Thus when an angular shape of actively growing mycelium is inserted into an agar

---

*The length of the growth unit in *Botrytis* can be derived from measurements given by Smith (1924), the point A being in the region of log $2 \cdot 5$–$2 \cdot 7$ μ, i.e. the length of the growth unit is 300–500 μ, which is three to four times its length in *Chaetomium*; some observations made in connexion with the experiments reported in this paper have shown that the exponential phase in *Botrytis* continues until the radius of the colony is about 500 μ.

plate and grows freely, rate of expansion over the surface of the agar is the same whether the hyphae are advancing along a line or are spreading out radially about an angle.

In this paper so far the term "colony" has been used without qualification. Here we are concerned with organization in the mycelium, not with specialization, and the evidence presented indicates a rather loose dependence between the individual hyphae through the growth unit.

In conclusion, it is suggested that growth by apical elongation of a tubular hypha associated with exponential increase of the mass by branching, might represent an evolutionary pathway by which a single-celled organism could become a larger multinucleate organism.

VI. Acknowledgments

I am particularly indebted to Dr. Joan M. Nicolls who was associated with me in that part of the work reported in this paper which was carried out at the Physics Department, University of Tasmania (Plomley and Ford 1946).

I have also had the benefit of discussions with Prof. E. J. G. Pitman, University of Tasmania, with Prof. J. S. Turner, University of Melbourne, with Mr. J. Bailey, Mr. D. M. Griffin, Dr. B. R. A. O'Brien, Dr. B. L. Reid, and Prof. N. H. White, all of the University of Sydney, and with Dr. U. Mittwoch, Galton Laboratory, University College, London, and wish to thank them.

I would also like to express my thanks for the facilities for undertaking this work which were given to me in the Physics Department, University of Tasmania, and in the Department of Histology and Embryology and School of Agriculture, University of Sydney.

VII. References

Castle, E. S. (1940).—Discontinuous growth of single plant cells measured at short intervals, and the theory of intussuseption. *J. Cell. Comp. Physiol.* **15**: 285–98.

Hinshelwood, C. N. (1946).—"The Chemical Kinetics of the Bacterial Cell." (Oxford Univ. Press.)

Machlis, L. (1957).—Factors affecting the lag phase of growth of the filamentous fungus, *Allomyces macrogynus*. *Amer. J. Bot.* **44**: 113–19.

Plomley, N. J. B., and Ford, Joan M. (1946).—Colony formation in the fungus *Chaetomium globosum* Kunze. (In Plomley, N. J. B., M.Sc. Thesis, University of Tasmania.)

Smith, J. H. (1923).—On the apical growth of fungal hyphae. *Ann. Bot.* **37**: 341–3.

Smith, J. H. (1924).—On the early growth rate of the individual fungus hypha. *New Phytol.* **23**: 65–78.

STRAIN DIFFERENCES IN THE MORPHOLOGY OF THE GAMETES OF THE MOUSE

By A. W. H. Braden*

[*Manuscript received July* 17, 1958]

Summary

Characteristic differences in the morphology of the sperm head have been noted between the inbred mouse strains C57BL, CBA, A, and RIII. There were interstrain differences in the shape of the posterior portion of the sperm head and in the length and width of the head. The sperm of mice derived from certain interstrain crosses were also studied.

Using the same four inbred strains, interstrain variation was observed in the degree of aggregation of certain large cytoplasmic granules in freshly ovulated eggs. The morphology of eggs from F_1 females of interstrain crosses was found to be intermediate between those of the parent strains. In outbred animals, variation between eggs from the same female was noted.

The observations provide further evidence that the morphological characters of the gametes are, directly or indirectly, determined by the genotype of the animal. The time of gene action in relation to meiosis is discussed.

I. Introduction

It has long been known that marked differences exist between species in the morphological characteristics of the gametes. Within the order Rodentia great interspecies variation in the size of the sperm and in the shape of the sperm head has been shown to exist (Retzius 1909; Friend 1936). Differences between the egg diameter of rodent species (Austin and Bishop 1957) and in the type, number, and distribution of the cytoplasmic granules of the egg have also been described (e.g. Boyd and Hamilton 1952).

Reports of variation in the morphology of the gametes between breeds, strains, or individuals within a single species are largely restricted to the description of differences between individuals in the incidence or type of grossly abnormal sperm (e.g. Williams and Savage (1925) in the bull; Moench and Holt (1931*a*) in man; and Bryson (1944) in the mouse). Differences in their characteristic sperm morphology between fertile males have been reported in the bull (Williams and Savage 1925), man (Moench and Holt 1931*b*), and several species of rodents (Friend 1936). In each instance there was significant variation in the head lengths of sperm from different individuals of a given species. No report of intraspecies variation in egg morphology appears to have been published.

In the course of a study of fertilization in four inbred strains of mice (Braden 1957*b*, 1958*b*), consistent differences between strains in certain morphological

---

*Division of Animal Health and Production, C.S.I.R.O., Sheep Biology Laboratory, Prospect, N.S.W., and Institute of Animal Genetics, Edinburgh, Scotland.

characteristics of both eggs and sperm were noted. These differences have been studied and the findings are reported here. A preliminary account of the work has been given elsewhere (Braden 1957*a*).

II. Material and Methods

The mice used were from the inbred strains CBA_{Ca}, $C57BL_{Fa}$, A_{Fa}, $RIII_{Fa}$ (Carter *et al*. 1952) and from certain interstrain crosses. A total of over 4000 eggs were examined (Braden 1957*b*, 1958*b*). Sperm were obtained from the vas deferens of males immediately after killing and suspended in 0·85 per cent. NaCl solution. Using phase-contrast microscopy, the sperm were examined in the fresh state in a very thin film between coverslip and slide. Eggs were recovered from mated females by dissection of the fallopian tubes in 0·85 per cent. NaCl solution, and were immediately examined by phase-contrast microscopy after slight compression between coverslip and slide.

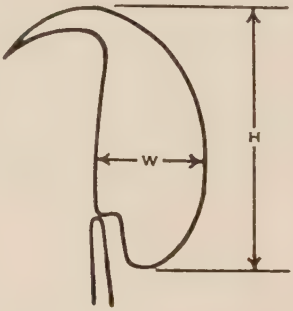


Fig. 1.—Illustrating the measurements made on photographs of sperm heads. *H*, maximal length; *W*, maximal width.

III. Results

(*a*) *Sperm*

When sperm from males of the strains CBA and C57BL were examined, it was immediately obvious that there was a strain difference in the shape of the posterior end of the sperm head. In sperm from C57BL males this part of the head was characteristically angular (Plate 1, Fig. 5) whereas in sperm from CBA males it was either smoothly rounded as in Plate 1, Figure 6, or slightly flattened as in Plate 1, Figure 7. About 2 per cent. of the sperm from CBA males exhibited the angular shape typical of sperm from C57BL males, but in 10–20 per cent. of sperm from C57BL males the posterior part of the head was flattened or rounded. Most of the sperm obtained from F_1 males produced from reciprocal crosses of the two strains resembled those of CBA males; the incidence of sperm with the angular type of head was approximately 10 per cent. In five back-cross males (C57BL ♀ × F_1 (CBA × C57BL) ♂) examined, the majority of the sperm were again of the CBA type, the proportion of sperms in which the posterior part of the head was angular in shape varying between 2 and 30 per cent.

In the inbred strains A and RIII the conformation of the posterior part of the sperm head was generally angular but not so markedly as in sperm from C57BL males (Plate 1, Figs. 8 and 9).

Measurements of the maximal length and maximal width of the sperm head were also made. The measurements were made on photographic prints made from negatives taken on 35-mm film at approximately 500 diameters. All the photographs were taken at exactly the same magnification. The measurements made are shown in Figure 1, and the results are presented in Table 1. The medium used for diluting the epididymal sperm (0·85 per cent. NaCl) caused some swelling of the acrosome (see Plate 1, Figs. 5–9) in most of the sperm, so the acrosome was not included in measurements of the length and width of the sperm head. It was subsequently learned that swelling of the acrosome may be prevented by inclusion of glucose in the medium (M. W. H. Bishop, personal communication). The sub-line CBA-*p* was characterized by the presence in homozygous condition of the gene "pink-eyed dilution" (symbol *p*) which appeared spontaneously in the strain CBA_{Ca} (Carter 1950).

TABLE 1

MEAN VALUES (WITH STANDARD ERRORS) FOR THE LENGTH AND WIDTH OF SPERM HEADS FROM VARIOUS STRAINS

Measurements made on photographs taken at constant magnification (*c.* ×2500)

Strain	No. of Males Used	No. of Sperm Measured	Mean Length (cm)	Mean Width (cm)
C57BL	3	23	$2\cdot15\pm0\cdot02$	$0\cdot91\pm0\cdot01$
CBA	4	24	$2\cdot36\pm0\cdot02$	$1\cdot03\pm0\cdot01$
Back cross CBA/C57	4	18	$2\cdot15\pm0\cdot03$	$0\cdot95\pm0\cdot01$
CBA-*p*	1	13	$2\cdot36\pm0\cdot03$	$1\cdot12\pm0\cdot02$
A	3	13	$2\cdot28\pm0\cdot02$	$1\cdot04\pm0\cdot01$
RIII	2	7	$2\cdot19\pm0\cdot03$	$1\cdot06\pm0\cdot01$

Analyses of variance of the results showed that the interstrain variation was statistically highly significant in respect of both head length ($P<0\cdot001$) and width ($P<0\cdot001$), but that the differences between males within a strain were not ($P<0\cdot1$). The sperm heads from CBA mice were both longer and wider than those from C57BL mice. The size of the sperm head in back-cross males was similar to that in C57BL males. The length of the sperm head in the sub-line CBA-*p* was similar to that of the strain from which it was derived, but the width of the sperm head was greater than in CBA. However, as the number of sperm heads measured was relatively small and only a few males were used, these findings must be treated with some reserve until a more extensive study of the subject can be made.

(*b*) *Eggs*

Great interstrain variation in the degree of aggregation of large cytoplasmic granules was noted in the eggs of the four inbred strains. Intrastrain and intra-animal variation was small. The granules were the largest type of granule seen in the eggs and were probably lipid in nature for they were refractile and birefringent, and did not stain with toluidine blue, Nile blue, or Janus green. They were evidently not identical with the "globules" described in fixed mouse eggs by Lams and Doorme (1908) and Gresson (1933), which were much larger in size and fewer in number.

The eggs from females of the CBA and C57BL strains (Plate 1, Figs. 1 and 2) were characterized by the presence of large aggregates of granules whereas there was no appreciable aggregation in the eggs of females from the RIII strains (Plate 1, Fig. 4); in A strain the granular aggregates were small (Plate 1, Fig. 3). Although the eggs from both CBA and C57BL females contained large aggregates of granules, the eggs of the two strains were readily distinguished from one another by a difference in the number of unaggregated granules: eggs from C57BL females had few such granules, whereas those from CBA females had many. A consequence of this difference was that in eggs undergoing fertilization the pronuclei were much more easily seen in eggs from C57BL females than in eggs from CBA females. The eggs of F_1 females from the crosses C57BL ♀×CBA ♂ and CBA ♀×C57BL ♂ resembled the CBA type rather than the C57BL type, but there was some variation between females and between eggs from the same female. In females derived from the back cross C57BL ♀×F_1 (C57BL×CBA) ♂ the number of unaggregated granules was intermediate between those of the parent lines CBA and C57BL. Eggs from females derived from the crosses RIII ♀×C57 ♂ and RIII ♀×A ♂ were also examined. The degree of aggregation of the granules in the eggs of these hybrids was found to fall between those characteristic of the parent lines, but there was more intra-animal variation than in females from the inbred strains.

In the freshly ovulated eggs of CBA and F_1 (CBA×C57) females, it was not uncommon to find the granular aggregates mainly concentrated in one hemisphere of the vitellus. In each of 20 such eggs that were closely studied the egg chromosomes or the early female pronucleus were found in the hemisphere containing the granules. Thirteen of these 20 eggs had already been penetrated by sperm; in 10 eggs the sperm head or early male pronucleus was located in the opposite hemisphere, and in three it was in the same hemisphere as the granular aggregates. This result is in contrast to the findings of Ludwig (1954) who reported that the site of attachment of the sperm head on 25 rat and 20 hamster eggs was twice as often in the hemisphere containing the egg chromosomes as in the opposite one. However, neither Ludwig's findings nor the present ones show a statistically significant departure from random distribution, and, as the two sets of results are in contradiction, it seems very likely that preferential attachment of the sperm head to a specific section of the vitelline surface does not occur. The uneven distribution of granular aggregates noted in eggs from CBA and F_1 (CBA×C57) females was apparently transient for it was not seen in eggs that had well-grown pronuclei. Furthermore, examination of eggs at various stages up to the eight-cell stage yielded no evidence of asymmetry in distribution of granules between blastomeres.

Primary oocytes, recovered from moderately large follicles in the ovaries of C57BL and A strain females about 12 hr before the expected time of ovulation, had many granules of the type described in tubal eggs, but the granules were not aggregated.

In examining the eggs from females of an outbred Swiss strain of mice in the course of other experiments great variation in the degree of aggregation of the granules was sometimes found among the eggs from a single female.

IV. DISCUSSION

Previous evidence of intraspecies variation in the morphology of the gametes of fertile individuals consists mainly of reports of significant differences in the head lengths of sperm from different males. Such variation has been found in man (Moench and Holt 1931*b*), the bull (Williams and Savage 1925), and four species of rodent: *Mus musculus*, *Rattus rattus*, *Apodemus sylvaticus*, and *Apodemus flavicolus* (Friend 1936). The variation in the phenotype of the sperm was thought to be the result of variation in genotype between the individual males used (Friend 1936), and the present findings provide further evidence in support of this conclusion.

The genes controlling the morphology of the sperm may act indirectly through the soma or directly in the developing sperm cell itself. Differentiation between "direct" and "indirect" gene action is extremely difficult except, possibly, when direct gene action takes place after meiosis and only one or two genes are involved. In the present study of sperm morphology, there was no indication of postmeiotic gene action but in certain other instances the evidence is suggestive of such action for more than one type of sperm was produced by individual males. Beatty (1956) found that the melanizing activity of rabbit semen in the presence of dihydroxyphenylalanine (DOPA) varied according to the coat colour of the male. The semen of males with a dark coat colour gave a DOPA-reaction, whereas the semen of males with light coat colour gave a weak reaction. When the semen of a heterozygous pale/dark male was examined, some of the sperm had melanizing activity and some had none, suggesting that the phenotype of any one sperm had been determined by its genotype after meiosis though no firm conclusion could be drawn. A postmeiotic gene action affecting sperm *function* is suggested in recent results obtained from mice carrying mutant alleles of the *t* series. In males carrying one *t* allele the transmission ratio of *t* and its wild-type allele is abnormal (Chesley and Dunn 1936; Dunn and Gluecksohn-Schoenheimer 1939; Dunn 1956), and this appears to be due to *t*-bearing sperm being more effective in fertilizing eggs than the sperm bearing the normal allele (Braden 1958*a*). Males that have two different *t* alleles are usually very infertile, but in this instance the gene action is evidently premeiotic (Dunn and Gluecksohn-Schoenheimer 1943; Bryson 1944; Braden and Gluecksohn-Waelsch 1958). The *t* alleles also have an effect on sperm morphology—they bring about an increase in the proportion of grossly abnormal sperm (Bryson 1944; Braden and Gluecksohn-Waelsch 1958).

Characteristic differences between strains in the morphology of the eggs have also been found in the present investigation. The strains differed in the degree of aggregation of certain cytoplasmic granules in ovulated eggs. As the granules were not aggregated when examined about 12 hr before ovulation it is possible that postmeiotic gene action is involved in this instance also. The first meiotic division is completed about 5 hr before ovulation in the rat (Odor 1955) and it is very likely that similar time relations apply in the mouse. The observation that an outbred female may produce eggs that differ markedly in the degree of aggregation of the granules supports the hypothesis of postmeiotic gene action, for all eggs from a female would have the same genetic constitution until the first meiosis had taken place.

The interstrain variations in the morphology of the gametes that have been described were rather obvious ones. It is very likely that there were many other less obvious differences between the strains in both the morphology and function of the gametes. Some evidence of variation between the gametes of different strains of mice and rats in the function of the gametes has been presented elsewhere (Braden 1957*b*, 1958*b*, 1958*c*). The findings indicate that the processes involved in the maturation and functioning of the gametes are under genetic control to a greater extent than has generally been recognized hitherto. They also suggest that investigations of the inheritance of the morphological or physiological characters of the gametes may be of value in the study of the mode of action of genes in mammals.

V. ACKNOWLEDGMENTS

The major part of this study was carried out during the tenure of a Studentship granted by the C.S.I.R.O., Australia, at the Institute of Animal Genetics, Edinburgh. I wish to express my thanks to the Director, Professor C. H. Waddington, F.R.S., for his hospitality and interest, and to Dr. R. A. Beatty for much help received during the course of the investigation. Thanks are also due to Dr. D. S. Falconer, Dr. T. C. Carter, and Dr. Ruth Clayton for mouse stocks. I am indebted to Mr. H. Weiler, Division of Mathematical Statistics, C.S.I.R.O., for the analyses of variance.

VI. REFERENCES

AUSTIN, C. R., and BISHOP, M. W. H. (1957).—Fertilization in mammals. *Biol. Rev.* **32**: 296–49.

BEATTY, R. A. (1956).—Melanizing activity of semen from rabbit males of different genotype. *Proc. R. Phys. Soc., Edin.* **25**: 39–44.

BOYD, J. D., and HAMILTON, W. J. (1952).—Cleavage, early development and implantation of the egg. In Marshall's "Physiology of Reproduction". 3rd Ed. Ch. 14. (Ed. A. S. Parkes.) (Longmans, Green & Co.: London.)

BRADEN, A. W. H. (1957*a*).—Differences between inbred strains of mice in the morphology of the gametes. *Anat. Rec.* **127**: 270–1.

BRADEN, A. W. H. (1957*b*).—Variation between strains in the incidence of various abnormalities of egg maturation and fertilization in the mouse. *J. Genet.* **55**: 476–86.

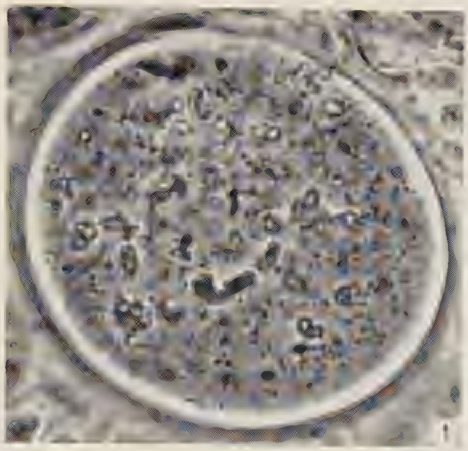
BRADEN, A. W. H. (1958*a*).—The influence of time of mating on the segregation ratio of alleles at the T locus in the house mouse. *Nature* **181**: 786–7.

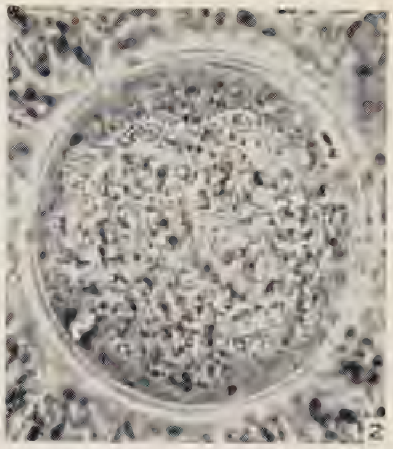
BRADEN, A. W. H. (1958*b*).—Variation between strains of mice in phenomena associated with sperm penetration and fertilization. *J. Genet.* **56** (in press).

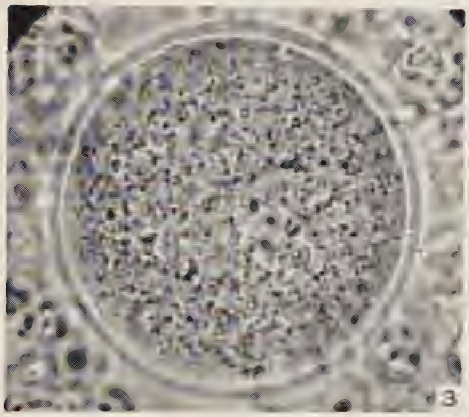
BRADEN, A. W. H. (1958*c*).—Strain differences in the incidence of polyspermia in rats after delayed mating. *Fertil. & Steril.* **9**: 243–6.

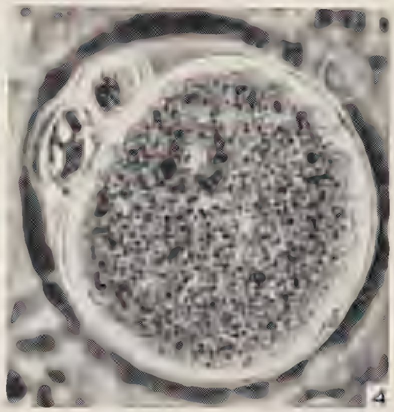
BRADEN, A. W. H., and GLUECKSOHN-WAELSCH, S. (1958).—Further studies of the effect of the T locus in the house mouse on male fertility. *J. Exp. Zool.* (in press).

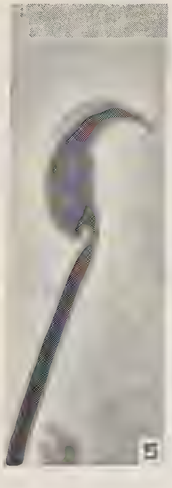
MORPHOLOGY OF MOUSE GAMETES

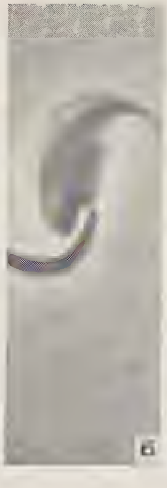


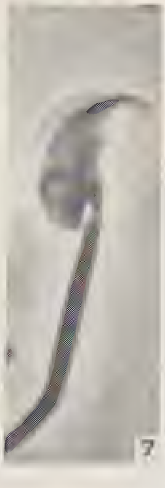


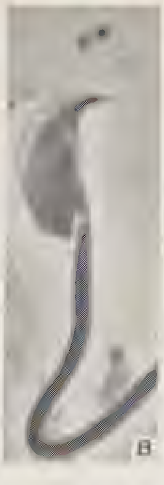


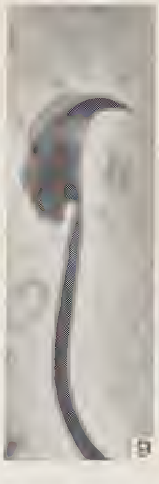












Bryson, V. (1944).—Spermatogenesis and fertility in *Mus musculus* as affected by factors at the T locus. *J. Morph.* **74**: 131–79.

Carter, T. C. (1950).—Mouse News Lett. No. 1. p. 2.

Carter, T. C., Dunn, L. C., Falconer, D. S., Grüneberg, H., Heston, W. E., and Snell, G. C. (1952).—Standardized nomenclature for inbred strains of mice. *Cancer Res.* **12**: 602–13.

Chesley, P., and Dunn, L. C. (1936).—The inheritance of taillessness (anury) in the house mouse. *Genetics.* **21**: 525–36.

Dunn, L. C. (1956).—Analysis of a complex gene in the house mouse. *Cold. Spr. Harb. Symp. Quant. Biol.* **21**: 187–95.

Dunn, L. C., and Gluecksohn-Schoenheimer, S. (1939).— The inheritance of taillessness in the house mouse. II. Taillessness in a second balanced lethal line. *Genetics* **24**: 587–609.

Dunn, L. C., and Gluecksohn-Schoenheimer, S. (1943).—Tests for recombination amongst three lethal mutations in the house mouse. *Genetics* **28**: 29–40.

Friend, G. F. (1936).—The sperms of the British Muridae. *Quart. J. Micr. Sci.* **78**: 419–43.

Gresson, R. A. R. (1933).—A study of the cytoplasmic inclusions and nucleolar phenomena during oogenesis in the mouse. *Quart. J. Micr. Sci.* **75**: 697–721.

Lams, H., and Doorme, J. (1908).—Nouvelles recherches sur la maturation et la fécondation de l'oeuf des Mammifères. *Arch. Biol., Paris* **23**: 259–366.

Ludwig, K. S. (1954).—Das Verhalten der Thymonukleinsäure (DNA) während der Befruchtung und den ersten Segmentationsstadien bei der Ratte und dem Goldhamster. *Arch. Biol., Paris* **65**: 135–78.

Moench, G. L., and Holt, H. (1931*a*).—Sperm morphology in relation to fertility. *Amer. J. Obstet. Gynec.* **22**: 199–210.

Moench, G. L., and Holt, H. (1931*b*).—Biometrical studies of head lengths of human spermatozoa. *J. Lab. Clin. Med.* **17**: 297–316.

Odor, D. L. (1955).—The temporal relationship of the first maturation division of rat ova to the onset of heat. *Amer. J. Anat.* **97**: 461–92.

Retzius, G. (1909).—Die Spermien der Nagetiere. *Biol. Untersuch.* **14**: 133–62.

Williams, W. W., and Savage, A. (1925).—Observations of the seminal micropathology of bulls. *Cornell Vet.* **15**: 353–74.

Explanation of Plate 1

Phase-contrast microscopy of ovulated eggs and of sperm from the various strains. Variation in egg diameter is due to differences in the degree of compression by the coverslip

Fig. 1.—Ovulated egg from strain CBA female. ×360.

Fig. 2.—Ovulated egg from strain C57BL female. ×315.

Fig. 3.—Ovulated egg from strain A female. ×315.

Fig. 4.—Ovulated egg from strain RIII female. ×395.

Fig. 5.—Sperm from strain C57BL male. ×1900.

Figs. 6 and 7.—Sperm from strain CBA male. ×1900.

Fig. 8.—Sperm from strain A male. ×1900.

Fig. 9.—Sperm from strain RIII male. ×1900.

THE PRE-NATAL DEVELOPMENT OF SKIN AND HAIR IN CATTLE (*BOS TAURUS* L.)

By A. G. Lyne* and Margaret J. Heideman*

[*Manuscript received August* 21, 1958]

Summary

A histological study has been made of the development of the skin and hair follicles in 33 foetuses, ranging in age from 68 to 274 days, and seven new-born specimens, of various breeds of *Bos taurus* L. Skin samples were taken from the mid-lateral region of the trunk, and both serial sections and whole mount preparations have been examined. Extensive use has been made of counts of the follicle and hair population as well as skin and hair follicle measurements which hitherto have apparently received little attention in studies of this type.

Follicle initiation extends from about the 77th day of gestation to about the 166th day. First-, later-, and last-formed follicle types are recognized and their special features and rates of development are described. Since all the follicles are associated with sweat glands and nearly all with arrector pili muscles, no attempt was made to classify them into primary and secondary types similar to those described in sheep and other mammals. The hairs of the first-formed follicles emerge at about the 200th day, and 20 days later most of the follicles have emerging hairs. In one foetus (251 days) and two of the new-born calves there are a few second-cycle follicles with emerging hairs.

Branching of follicles as found in sheep and other mammals has not been observed. There is, however, the phenomenon of pairing, apparently not previously described in mammals, where two (occasionally three) follicles, arising independently from the epidermis, share a common hair canal.

Follicle group formation, although sometimes recognized, especially below the level of the sebaceous glands, is not a prominent feature in cattle.

Some features in the development of skin and hair follicles in *B. taurus* are compared with those in the sheep, mouse, and other mammals. In *B. taurus* the maximum follicle density of 233 per mm^2 at approximately 166 days gestation decreases to between 46 and 88 per mm^2 at birth, when the hair density is even slightly greater than in Merino sheep at the same conception age (i.e. approximately 4 months after birth).

Attention is drawn to the need for further pre- and post-natal histological studies of the integument of different breeds of cattle, and particularly of seasonal and nutritional influences on the hair cycles. Such studies are fundamental to a better understanding of the variations in the coat associated with acclimatization.

I. INTRODUCTION

Although cattle are economically very important, there are comparatively few publications on the skin and coat. For the most part, these studies have been concerned with the importance of the coat in problems of acclimatization and

---

*Division of Animal Health and Production, C.S.I.R.O., Sheep Biology Laboratory, Prospect, N.S.W.

environmental physiology (Camek 1920; Duerden and Whitnall 1930; Bonsma and Pretorius 1943; French 1946; Bonsma 1949; Yeates 1954, 1955, and 1958). In these studies particular emphasis has been given to the types of hair, and the influence of season, environment, and nutrition.

In addition, a number of histological investigations have been made. The structure, and in some cases the density, of the hair follicles and their associated structures, has been studied by de Meijere (1894), Yamane and Ono (1936), Barisson Villares and Berthet (1951*a*), Carter and Dowling (1954), Dowling (1955*a*, 1955*b*), Hafez, Badreldin, and Shafei (1955*a*, 1955*b*), and Walker (1957*b*). Other work, directed particularly to the structural and functional aspects of sweat glands, has been carried out by Findlay and Yang (1950), Barrison Villares and Berthet (1951*b*), Goodall and Yang (1952), Yang (1952), Ferguson and Dowling (1955), Nay and Hayman (1956), and Nay and Dowling (1957). Furthermore, Findlay and Yang (1948) and Goodall and Yang (1954) investigated capillary distribution in the skin, and Dowling (1955*b*), Walker (1957*a*), and Patel and Anderson (1958) measured skin thickness. The majority of these observations were made on adult material.

All authors agree that in the cow there is only one type of follicle, similar to the primary follicle of sheep in that it is associated with a sweat gland, sebaceous gland, and arrector pili muscle. However, distinctly larger follicles with thicker and larger hairs occur amongst the smaller ones. This, and not the usual criterion for follicle classification which is based on the position in the follicle group and the presence or absence of some of the associated structures, forms the basis of Bonsma's (1949) division of the hairs into primary and secondary types.

With the exception of Postma (1947), who used the stage of hair follicle development as seen with the naked eye as one of the criteria for estimating foetal age, and Winters, Green, and Comstock (1942), who made a few observations, there seems to be no published account of the development of the skin and hair follicles in cattle. It is the aim of this paper to give such an account of the pre-natal development in *Bos taurus* L., and to make a comparison of some features with those of other mammals.

II. MATERIAL AND METHODS

(*a*) *Animals and Age Determination*

Details of the animals sampled are shown in Table 1. Twenty-five of the foetuses were collected from freshly killed animals at the State Abattoir, Homebush, N.S.W., and the remainder of the material from eastern New South Wales.

The body weights of all specimens were obtained and body measurements were made of all but seven foetuses and four new-born calves.

The foetal age was determined from information available on the artificial insemination or last service date of the dam. The recorded age of each foetus was checked against Postma's (1947) body weight and body length data for black and white and red and white cattle in Holland. An additional check of foetal age was carried out using the data of Winters, Green, and Comstock (1942). When the estimated age differed markedly from the recorded age the former was used.

TABLE 1

DETAILS OF B. TAURUS SPECIMENS SAMPLED

Foetal Age (days)	Sex	Breed	Foetal Age (days)	Sex	Breed
68 (a)	♀	Holstein–Friesian crossbred	188	♂	Ayrshire crossbred
68 (b)	♀	Australian Illawarra Shorthorn × Ayrshire	203	♀	Guernsey × Jersey
74	♀	Holstein–Friesian crossbred	209	♂	Australian Illawarra Shorthorn
77	♀	Australian Illawarra Shorthorn	218 (a)	♂	Holstein–Friesian
84	♂	Holstein–Friesian ?	218 (b)	♀	Guernsey × Jersey
87*(a)	♀	Holstein–Friesian ?	248	♂	Guernsey
87*(b)	♂		251	♀	Ayrshire crossbred
88	♀	Australian Illawarra Shorthorn × Shorthorn	253	♀	Australian Illawarra Shorthorn × Shorthorn
91†	♀	Australian Illawarra Shorthorn × Guernsey	255	♀	Australian Illawarra Shorthorn
103	♀	Holstein–Friesian × Ayrshire	258	♀	Jersey
119	♀	Australian Illawarra Shorthorn	260	♀	Australian Illawarra Shorthorn × Shorthorn
134	♀	Australian Illawarra Shorthorn × Shorthorn	266†	♂	Holstein–Friesian crossbred
147†	♂	Guernsey × Jersey	274	♂	Jersey
149	♂	Holstein–Friesian crossbred	Birth (a)	♂	Ayrshire
161	♀	Guernsey	Birth (b)	♀	Jersey
166	♂	Holstein–Friesian crossbred	Birth (c)	♀	Jersey
178	♀	Holstein–Friesian crossbred	Birth (d)	♀	Jersey
179	♀	Holstein–Friesian crossbred	Birth (e)	♀	Jersey
180	♂	Holstein–Friesian	Birth (f)	♂	Australian Illawarra Shorthorn × (A.I.S. × Shorthorn)
183†	♂	Guernsey × (Guernsey × Jersey)	Birth (g)	♂	Ayrshire

*Twins. †Age estimated.

(b) *Skin Sampling and Histological Methods*

All skin samples were taken from the mid-lateral region of the trunk. At least two samples were taken from each specimen and one or both of these were fixed in 5 per cent. formol saline. Some additional samples were fixed in Zenker's or Bouin's fixative.

With the exception of six foetuses, at least one sample from each specimen was taken with a trephine, 1 cm in diameter, similar to that used by Carter and Dowling (1954), and serial-sectioned at 8 μ parallel to the skin surface. Some additional rectangular skin samples were taken for vertical serial sections of the skin cut at 8, 15 or 20, and 40 μ, parallel to the long axes of the hair follicles. All the sections were stained with haemalum, eosin, and picric acid. Haemalum-stained whole mounts of skin of foetuses up to 119 days were also examined.

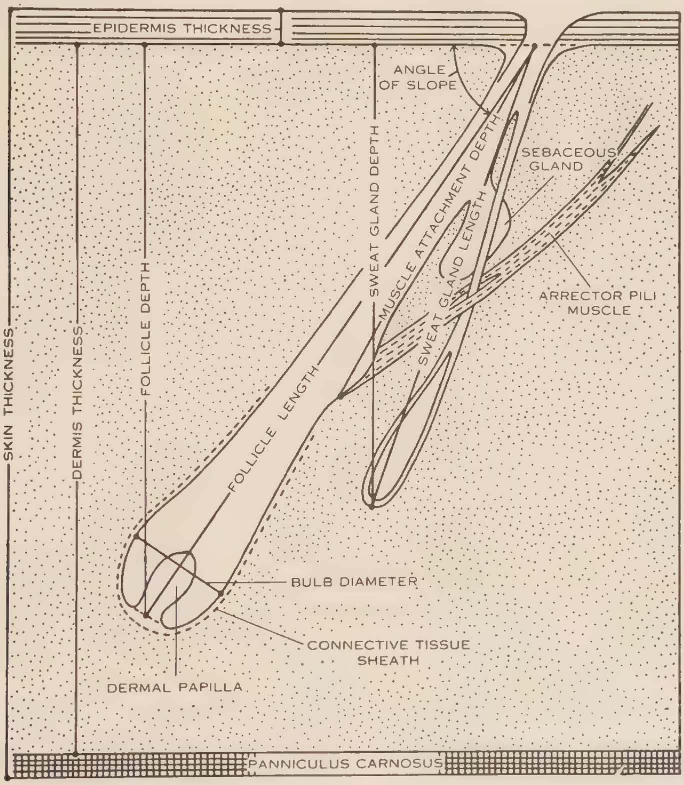


Fig. 1.—Diagrammatic vertical section of the skin to show the method of taking measurements, all of which are straight.

(c) *Skin and Follicle Measurements*

(i) *Macroscopic Measurements*.—The total thickness of the trephined skin sample, or of an additional sample taken from the preserved foetus or complete skin, was measured with skin callipers under constant pressure. Where necessary, hair was closely clipped, and any fragments of fascia on the subcutaneous surface were trimmed to the panniculus carnosus.

(ii) *Microscopic Measurements.*—The manner in which the microscopic measurements of the skin, hair follicles, and associated structures were taken is illustrated in Figure 1. These measurements were made on all complete follicles, irrespective of type or size, using a microprojector at a magnification of ×215 or ×500.

The counts of follicle and hair density were made with a microprojector at a magnification of × 115 or × 215, or both, on two to eight fields each of 1 mm^2 (see Table 2), taken at random on whole mounts or transverse sections of skin, and corrected for shrinkage in a manner similar to that described by Carter and Dowling (1954).

(*d*) *Terminology*

As the formation of distinct follicle groups is not a characteristic feature of the skin of cattle, and because all follicles are associated with a sweat gland and usually an arrector pili muscle, no attempt has been made to classify the follicles into primary and secondary types similar to those recognized in sheep (Hardy and Lyne 1956) and bandicoots (Lyne 1957*a*).

In the place of this subdivision the follicles have been divided into first-, later-, and last-formed types. The *first-formed*, as well as being the largest pelage follicles in the foetus, are characterized by the formation of an ental swelling to which the arrector pili muscle is attached. Medullated hairs are formed by these follicles, at least during the first hair cycle. *Later-formed* follicles are characterized by their tendency to form pairs which share a common epidermal hair canal. The larger of the pair resembles the first-formed type in most features. The smaller does not form an ental swelling and its arrector pili muscle is very small, or occasionally may be absent, at least during the first hair cycle. Most of the later-formed follicles form non-medullated hairs. The *last-formed* include some of the smaller of the paired follicles in addition to solitary ones. Only non-medullated hairs are formed by these follicles during the first hair cycle.

III. THE DEVELOPMENT OF INDIVIDUAL FOLLICLES

(*a*) *Stages in the Development of Follicles of the First Hair Cycle*

The eight stages of hair follicle development for mammals in general (Hardy and Lyne 1956) can be recognized in *B. taurus*. The following account is limited to the special features of the follicles at each of the defined mammalian stages. Two subsequent stages have been added to cover the entire hair cycle, including the period of quiescence.

(i) *First-formed Follicles* (*see Fig.* 2)

The various stages are:

Stage 1: *Follicle plug.*—This stage is divided into 1*a*, when the length of the plug is less than its diameter (Plate 1, Fig. 1), and 1*b*, when the length is equal to or greater than the diameter.

Stage 2: *Pre-papilla.*—The base of the epidermal plug flattens, usually when its length is more than twice its diameter (Plate 1, Figs. 2 and 3). Soon after the flattening, the sweat gland rudiment appears as a solid bud from the ental side of the follicle at a high level (stage 2*a*). Late in this substage the first trace of the arrector pili muscle appears in the dermis and a swelling may be recognized about half-way down the ental side of the follicle. The sebaceous gland bud with differentiated cells is formed immediately below the junction of the sweat gland duct with

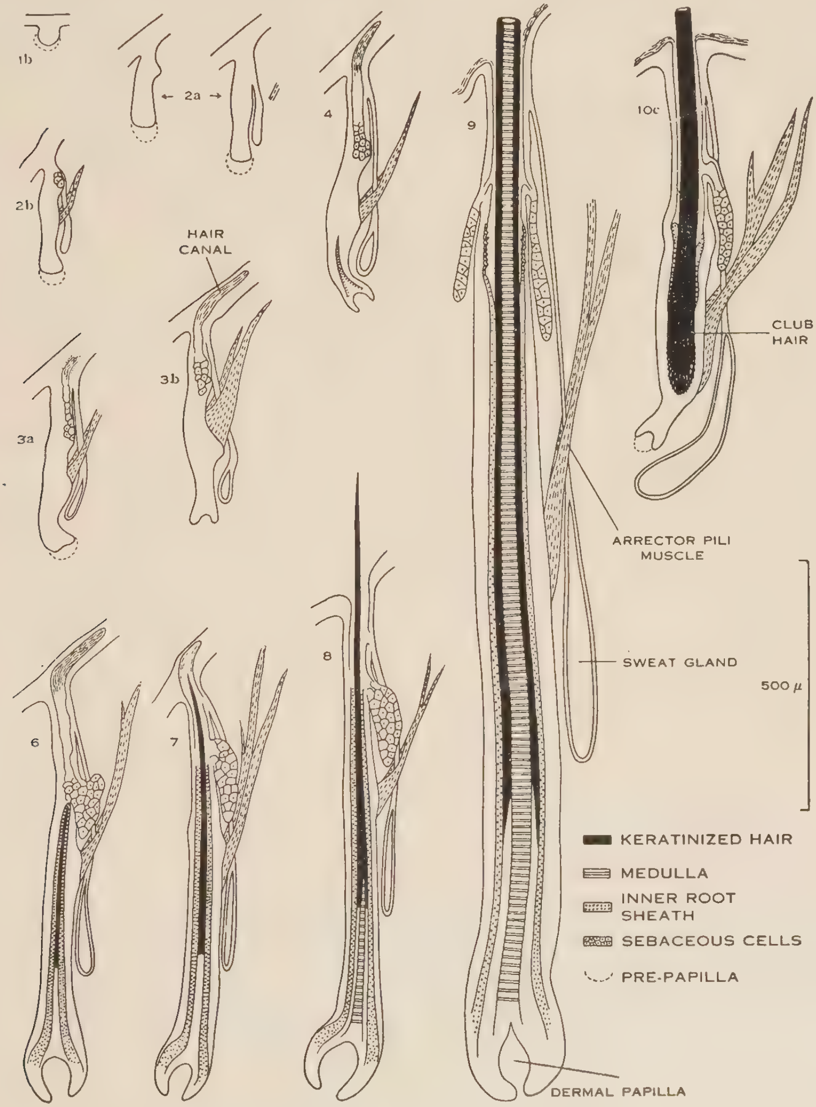


Fig. 2.—Diagram from camera lucida drawings of stages 1–10 in development of first-formed hair follicles in *B. taurus*.

the follicle (stage 2*b*). The first stage in the formation of the epidermal portion of the hair canal may be recognized.

Stage 3: *Papilla.*—This stage is subdivided according to the shape of the dermal papilla. At stage 3*a* the base of the follicle becomes concave and the dermal papilla is recognized; its depth is less than its diameter. The arrector pili muscle (usually of two strands) extends obliquely from the upper level of the dermis to the ental swelling which is now very prominent (Plate 1, Fig. 4). The hair canal is formed by the keratinization of epidermal cells and the migration and degeneration of sebaceous cells. The stages of hair canal development appear to be similar to those described in the Merino sheep foetus (Lyne 1957*b*). The sweat gland, which extends to a level between the ental swelling and follicle bulb, contains a small lumen. At stage 3*b* the length of the dermal papilla is at least equal to its diameter. The epidermal portion of the hair canal is bent so that it lies almost parallel to the skin surface. The very prominent ental swelling is at its maximum size (Plate 1, Fig. 5).

Stage 4: *Hair cone.*—The ental swelling is slightly less prominent (Plate 1, Fig. 6).

Stage 5: *Advanced hair cone.*—The tip of the hair cone is refractile and within it Huxley's layer and the developing hair cuticle and cortex are discernible. This differs from the general mammalian stage 5, as a keratinized hair may be present by the time the hair cone reaches the sebaceous gland level.

Stage 6: *Hair formation.*—The tip of a keratinized hair is recognized inside the hair cone which lies at the level of the sebaceous gland. All the follicle layers can be recognized.

Stage 7: *Hair in canal.*—The tip of the hair has emerged through the inner root sheath layers and entered the hair canal.

Stage 8: *Hair emerged.*—See Plate 2, Figure 1. Usually a flattened ental swelling can be recognized; occasionally it extends around to the ectal side of the follicle.

Stage 9: *End of follicle length growth.*—The follicle reaches it maximum length (Plate 2, Fig. 2; Plate 3, Fig. 1).

Stage 10: *Club hair formation.*—Three substages are recognized: stage 10*a*, when the follicle shows the first indication of cessation of activity (contraction of the bulb and wrinkling of the connective tissue sheath around it); stage 10*b*, when the formation of a club hair with a brush end is first recognized; and stage 10*c*, when the club hair is fully keratinized (Plate 2, Fig. 3; Plate 3, Fig. 2). Stages 10*a*–10*c* are accompanied by marked changes in the follicle and hair. Medulla formation ceases, followed by cessation of the formation of hair cuticle and inner root sheath. Meanwhile the follicle decreases in length and the bulb and dermal papilla degenerate until they appear somewhat as they did at stage 3.

(ii) *Later-formed Follicles*

(1) *Solitary Follicles.*—These follicles go through the same stages of development as the first-formed but they are smaller and an ental swelling is not recognized except in association with the largest in this category.

(2) *Paired Follicles* (*see Fig.* 3).—Two (occasionally three) follicles develop in juxtaposition directly from the epidermis and share a common hair canal which begins its development within the stratum spinosum (Plate 4, Figs. 1 and 2). The two follicles differ in size; the smaller and later-formed one is usually on the ectal side of the larger. Each forms a normal sweat and sebaceous gland and the two hairs which develop emerge through a common orifice (Plate 4, Fig. 3). If there is a size difference in the two hairs the smaller one is formed by the smaller follicle. The larger follicle has an arrector pili muscle and sometimes an ental swelling, whereas the smaller has only a muscle which is very small and may even be absent. The smaller follicle begins its development when the larger one is at about stage 2*a*, and when the latter is at stages 3*b* and 4 the former is usually at 2*a* and 3*b* respectively. The development of the smaller follicle may be very slow and in some specimens it appears to be vestigial. The larger follicle may reach stage 10*b* before the smaller one has reached stage 4. This suggests that some of the smaller follicles remain stationary during the active phase of the larger of the pair.

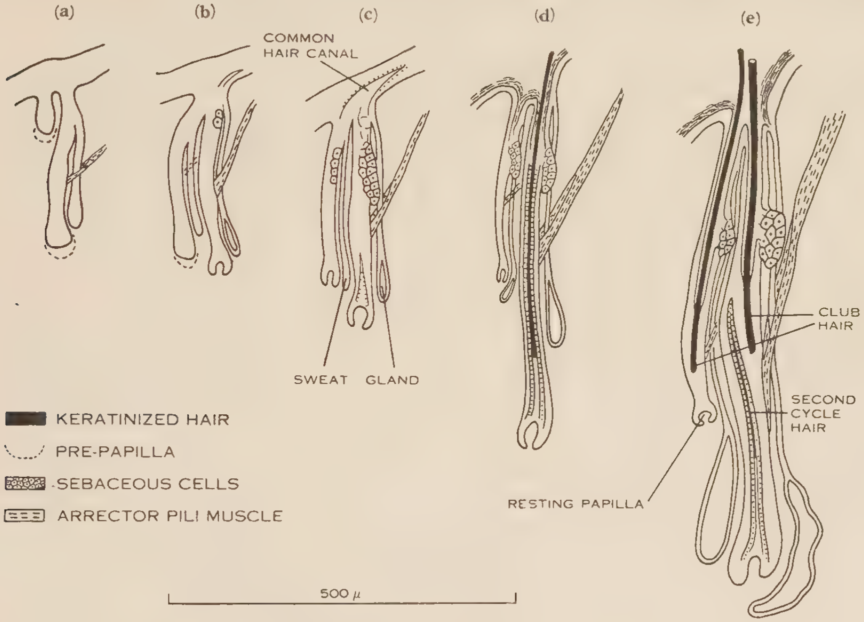


Fig. 3.—Diagram from camera lucida drawings of some stages in development of paired hair follicles in *B. taurus*.

(iii) *Last-formed Follicles*

Both solitary and the smaller of some of the paired follicles are included in this category and their development is similar to that described above. No ental swelling has been observed with these last-formed follicles.

(*b*) *Stages in the Development of Follicles of the Second Hair Cycle*

(i) *First- and Last-formed Follicles.*—In the material examined these follicles have not commenced the second hair cycle.

(ii) *Later-formed Follicles.*—Some of these follicles overtake the first formed during the first hair cycle. At stage 10*c* regeneration takes place from the base of the resting follicle. The cells of the hair germ proliferate and when a new hair cone is formed a stage is reached which is equivalent to stage 4 of the first hair cycle. The new hair usually grows up the follicle on the ectal side of the old hair. Regeneration of both solitary and the larger of some of the paired follicles has been observed in the material examined.

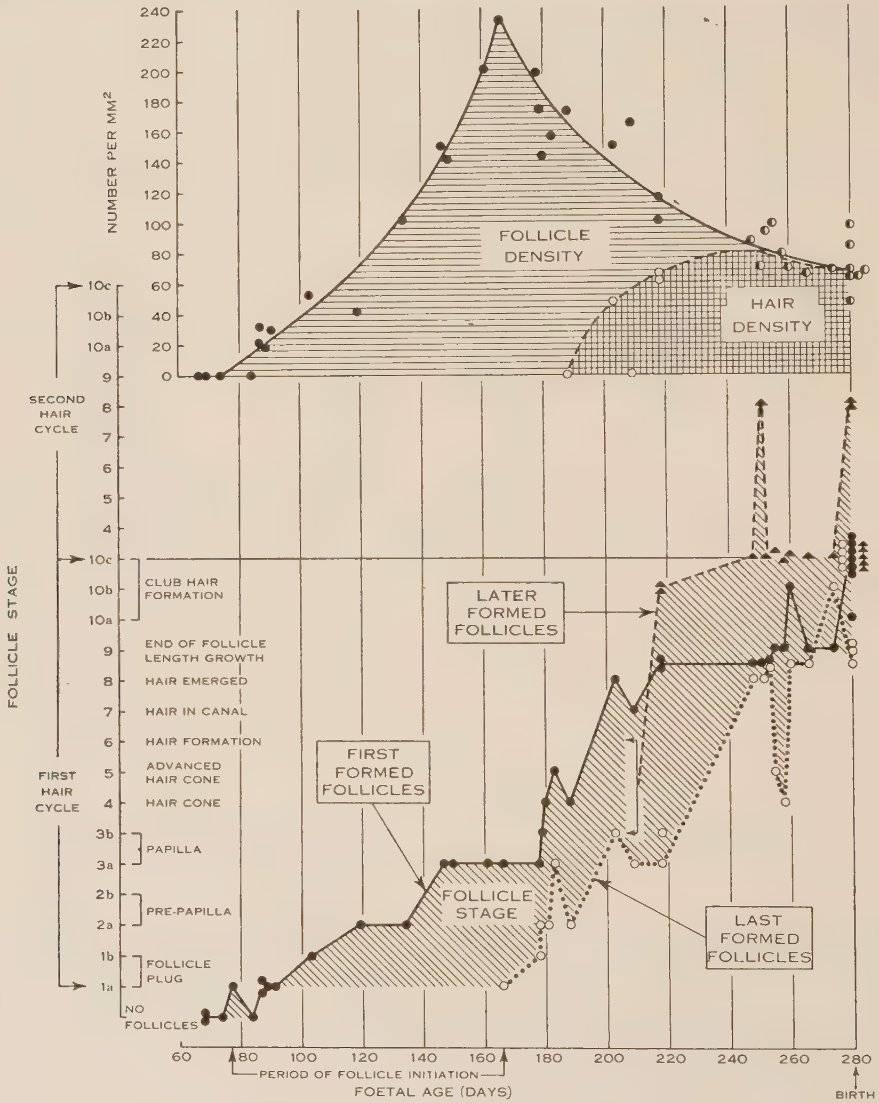


Fig. 4.—Relation between foetal age, stage of development of different types of hair follicles (most advanced of the first- and later-formed follicles; least advanced of the last formed), and the follicle and hair density in *B. taurus*. The curves through the follicle and hair density points have been drawn by free-hand.

(c) *Rate of Development of the Follicles*

(i) *First Cycle.*—The relation between the foetal age and the stage of development of the different types of follicles, and the period of follicle initiation (lasting about 3 months), are shown in Figure 4. As no hair follicles are formed by branching, *B. taurus* has its full complement of follicles more than 3 months before birth.

The most advanced of the first-formed follicles, which begin their development at about the 77th day of gestation, reached stage 8 in about 126 days and stage 10*c* in about 200 days. These first-formed follicles appear to remain stationary at stage 3*a* for about 30 days. As seen in Plate 2, Figures 2 and 3, there is a distinct difference in the stage reached by the follicles at birth in different specimens. In the four new-born Jerseys, all the follicles are at stage 10*c*, whereas in the remaining new-born material the first-formed follicles are at stages 9–10*c*, the most advanced of the later formed at stage 10*c* of the first hair cycle to stage 8 of the second hair cycle, and the last formed at stages 8–9.

Between 203 and 218 days some of the later-formed follicles overtake the first formed and some of them reach stage 10*b* by the end of this period.

Most of the last-formed follicles develop at a faster rate than the first formed, taking only about 120 days to reach stage 10*c* whereas the latter take 200 days. At 218 days, however, a few of the last-formed type appear vestigial and may not form a hair. At birth, all the follicles examined have reached at least stage 8 of the first hair cycle.

(ii) *Second Cycle.*—Some of the later-formed follicles have entered the second cycle at or before birth. In the 251-day foetus and in two of the seven new-born calves some second-cycle follicles have emerging hairs (Fig. 4).

(d) *Relation between the Foetal Age and the Follicle and Sweat Gland Length*

(i) *Follicle Length.*—The relation between the foetal age and the length of all the first hair cycle follicles measured is shown in Figure 5.

At 203 days, when the most advanced of the first-formed follicles are at stage 8, the maximum length is 1000 μ (Plate 2, Fig. 1). At 248–266 days some of these follicles have reached their maximum length of 2100–2200 μ. At birth the maximum length is 1600 μ. The later-formed follicles which overtake the first formed in stage of development, are much smaller and do not grow to the same length. The growth of the shortest of the last-formed follicles, as seen by joining the minimum points (Fig. 5), is markedly different to that of the longest of the first formed.

(ii) *Sweat Gland Length.*—The relation between the foetal age and the length of all the sweat glands measured is shown in Figure 6. After the maximum length is reached at approximately 250 days, there seems to be little change in length, other than that associated with the follicle stage. Insufficient material is available to ascertain any breed differences.

(e) *Relation between the Follicle Stage and the Follicle and Sweat Gland Measurements during the First Hair Cycle*

(i) *Follicle Length.*—When the maximum and minimum follicle length is plotted against the stage of development (Fig. 7), it is clearly seen that the elongation of

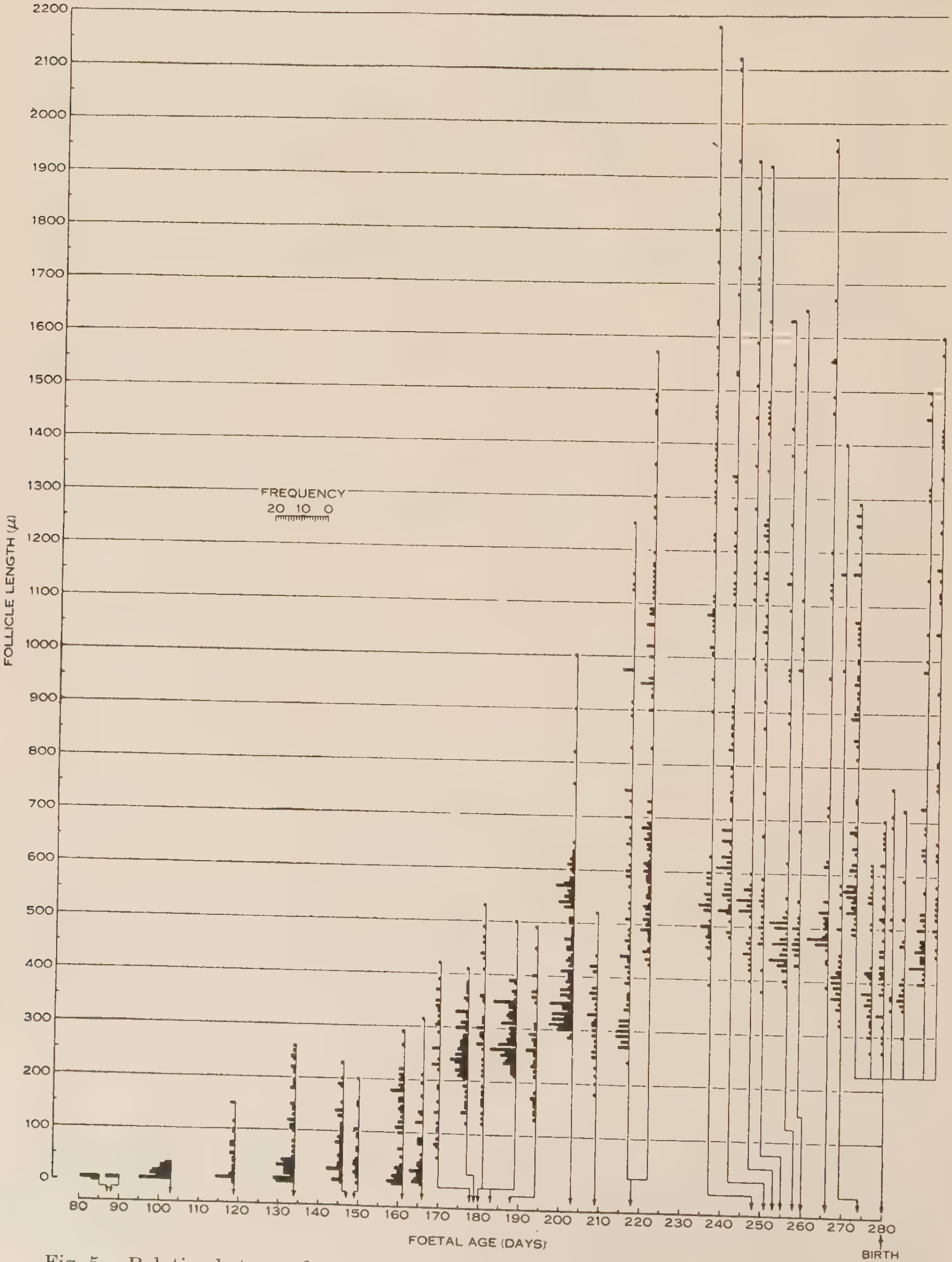


Fig. 5.—Relation between foetal age and the length of all follicles measured in *B. taurus*.

the longest follicles (first formed) is followed by a marked decrease in their length. The longest stage 9 follicles measured are more than $2\frac{1}{2}$ times the length of the longest at stage 10*c*. All the follicles reach a maximum length at stage 9 but this is not so prominent a feature in the shortest ones.

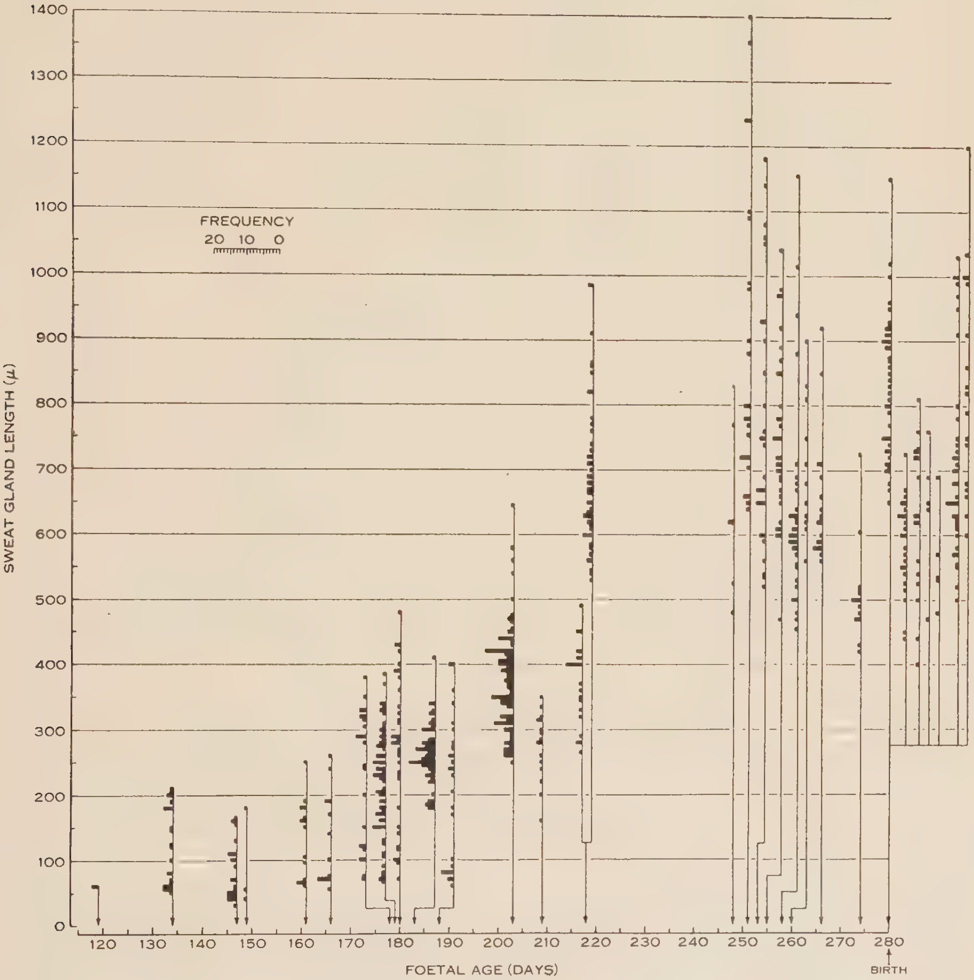


Fig. 6.—Relation between foetal age and the length of all sweat glands measured in *B. taurus*.

(ii) *Depth of Muscle Attachment*.—The depth of the muscle attachment (Fig. 7) also increases and then decreases during the first hair cycle. When the first-formed follicles are at stages 3*a*–4 the arrector pili muscle appears to be firmly attached to the ental swelling of the outer root sheath (Plate 1, Figs. 4 and 5). If this attachment remains, and as there appear to be very few cell divisions above it, it is possible that a large proportion of the increase in the length of the follicle is brought about by hypertrophy of the outer root sheath cells above the attachment.

(iii) *Bulb Diameter*.—The changes in the diameter of the bulb are similar to those for the length of the follicle except that it remains nearly unchanged at about 50 μ from stage 1 to stage 6. A maximum of approximately 200 μ is reached at stage 9, decreasing to approximately 50 μ at stage 10*c* when the follicle is quiescent.

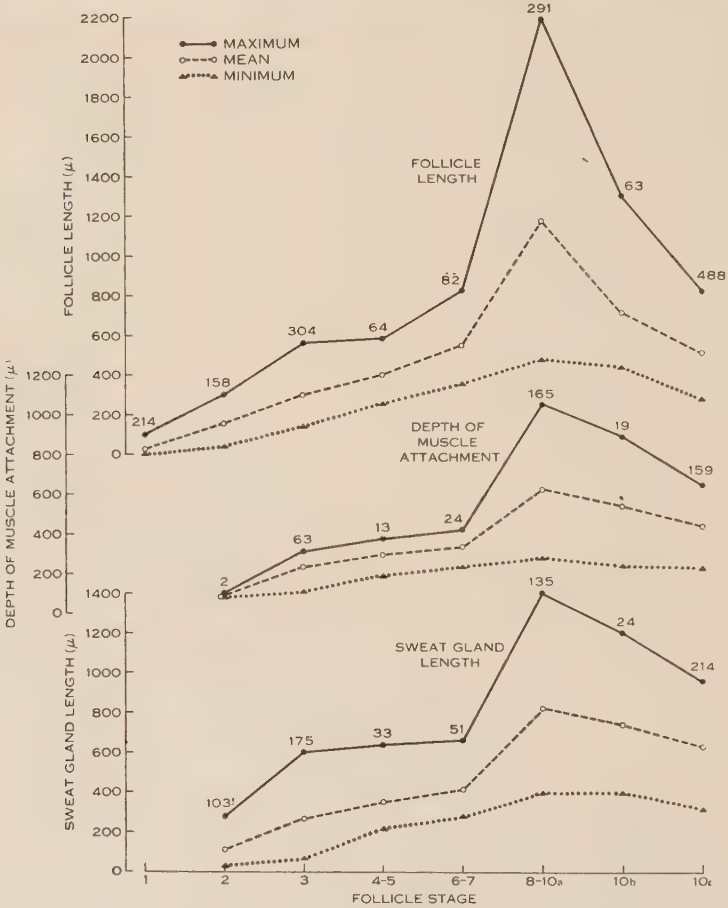


Fig. 7.—Relation between follicle stage, the maximum, mean, and minimum follicle length, depth of muscle attachment, and sweat gland length in *B. taurus*. The number measured is shown above the maximum points.

(iv) *Sweat Gland Length*.—The relation between the follicle stage and the maximum and minimum sweat gland length is shown in Figure 7. A maximum is reached at stage 9, decreasing slightly to stage 10*c*.

(v) *Angle of Slope*.—At stage 1*a* the follicle has no visible slant or slope (Plate 1, Fig. 1), although it typically develops one very shortly thereafter. In most cases, after the slope has become established the follicle continues to grow in the same direction. The angle of slope of the large first-formed follicles, however, changes with the stage of development of the follicle, reaching a minimum at stage 9. When the first hairs emerge there is a marked difference between the angle of slope of the first-formed follicles and the remainder (see Plate 2, Fig. 1). Later, when the first-formed follicles are at stage 9 there is less difference in this angle. As the follicles decrease in length in passing from the active to the quiescent stage they lie more perpendicularly to the skin surface (Plate 2, Fig. 3).

IV. Development of the Follicle and Hair Population

(*a*) *Follicle Groups*

The development of follicle groups, characteristic of most mammals, is not a prominent feature of the skin of cattle. However, group formation is not entirely absent at certain stages during the development of the follicle population.

The first hair follicles appear as isolated structures without any apparent order (Plate 5, Fig. 1). However, these follicles are not located at random with respect to one another; two individuals never touch one another. They are spaced one or more of their own diameters apart. Each of these first-formed follicles will be the central member of a different follicle group. As soon as each first-formed follicle has penetrated a short distance into the dermis it apparently acts as an organizer of other follicles close by, for a pair of satellites (later-formed follicles) then usually appears, the members of the pair taking positions on opposite sides of the central follicle (Plate 5, Fig. 2). When this trio stage is reached the main foundations of the follicle group have been laid.

After the formation of these trio groups more follicles (later and last formed) develop, usually on the ectal side of the group (Plate 5, Fig. 4). Some of these follicles form pairs, either with one another or with a lateral member of the trio, the smaller of the pair generally on the ectal side (Plate 5, Figs. 5 and 6).

(*b*) *The Density of the Follicles and Hairs*

The total population of follicles and keratinized hairs was counted on serial sections, cut parallel to the skin surface, and on stained whole mounts of skin. A summary of the results is shown in Table 2 and plotted in Figure 4. It is interesting to note that the maximum follicle density, which is reached at approximately 166 days gestation, coincides with the end of the period of follicle initiation from the epidermis. Thus, up to this stage, follicle formation more than balances skin expansion. The maximum of about 233 follicles per mm^2 (Plate 5, Fig. 3) decreases to between 46 and 88 follicles per mm^2 at birth (Plate 5, Fig. 6). This decrease in follicle density is proportional to the increase in surface area as calculated from the relation given by Brody (1945, p. 359) for Jersey and Holstein cattle:

$$A = 0{\cdot}15W^{0{\cdot}56},$$

where A = skin surface area in sq. m., and

W = body weight in kg.

The hair density reaches a maximum at about 255 days.

The slight differences in the follicle and hair densities after 248 days, as shown in Table 2, are due to the presence of some immature follicles (i.e. less than stage 6),

Table 2

SUMMARY OF STAGES IN THE DEVELOPMENT OF FIRST-FORMED FOLLICLES AND THE FOLLICLE AND HAIR POPULATION IN B. TAURUS

Foetal Age (days)	Most Advanced Stage of First-formed Follicles	No. of Fields Counted (of area 1 mm^2)	No. of Follicles per Field	No. of Hairs per Field	Percentage of Medullated Hairs as Seen in Sections
Whole mounts					
68(a)	—	—	—	—	—
68(b)	—	—	—	—	—
74	—	—	—	—	—
77	1*a*	—	?	—	—
84	—	—	—	—	—
87(a)	1*a*	2	31·4	—	—
87(b)	1*a*	4	20·3	—	—
88	1*a*	4	19·4	—	—
91	1*a*	5	29·3	—	—
103	1*b*	8	52·7	—	—
119	2*a*	8	41·4	—	—
Sections					
134	2*a*	6	101·1	—	—
147	3*a*	7	150·0	—	—
149	3*a*	3	140·8	—	—
161	3*a*	8	200·0	—	—
166	3*a*	8	233·3	—	—
178	3*a*	8	197·9	—	—
179	3*b*	6	173·9	—	—
180	4	8	143·3	—	—
183	5	8	156·0	—	—
188	4	3	173·1	—	—
203	8	8	150·4	47·4	?
209	7	4	164·7	0·3	—
218(a)	8–9	6	100·1	62·6	17·5
218(b)	8–9	6	115·6	65·0	12·1
248	8–9	6	87·7	86·0	21·2
251	8–9	6	69·3	71·2*	12·4
253	8–9	6	92·8	92·8	15·4
255	9	6	97·6	97·0	19·0
258	9	6	80·6	77·4	13·7
260	10*b*	6	69·5	68·2	10·4
266	9	6	64·3	64·3	12·2
274	9	6	67·9	67·8	9·3
Birth (a)	10*c*	8	67·2	66·8	17·2
Birth (b)	10*c*	8	81·1	81·1	(15·4)†
Birth (c)	10*c*	8	65·3	65·3	(14·5)†
Birth (d)	10*c*	7	60·9	60·9	(11·7)†
Birth (e)	10*c*	6	88·3	87·8	(14·0)†
Birth (f)	10*a*	5	65·1	65·0	15·3
Birth (g)	10*c*	6	46·1	46·1	16·8

*Includes second-cycle hairs.

†All club hairs (non-medullated). The percentage of large follicles is shown in parenthesis.

Winters, Green, and Comstock (1942) observed the first appearance of hair follicles in cattle in a 90-day foetus, and the complete covering of the body with hairs at 230 days. Macroscopic observations on the emergence of hair were also included in Postma's (1947) criteria for age determination of cattle foetuses. He states that both 210- and 212-day foetuses may be covered with short hair. Both these studies thus support present observations which show follicles on the mid-lateral region of the trunk first appearing at about 77 days and hairs emerging at about 203 days.

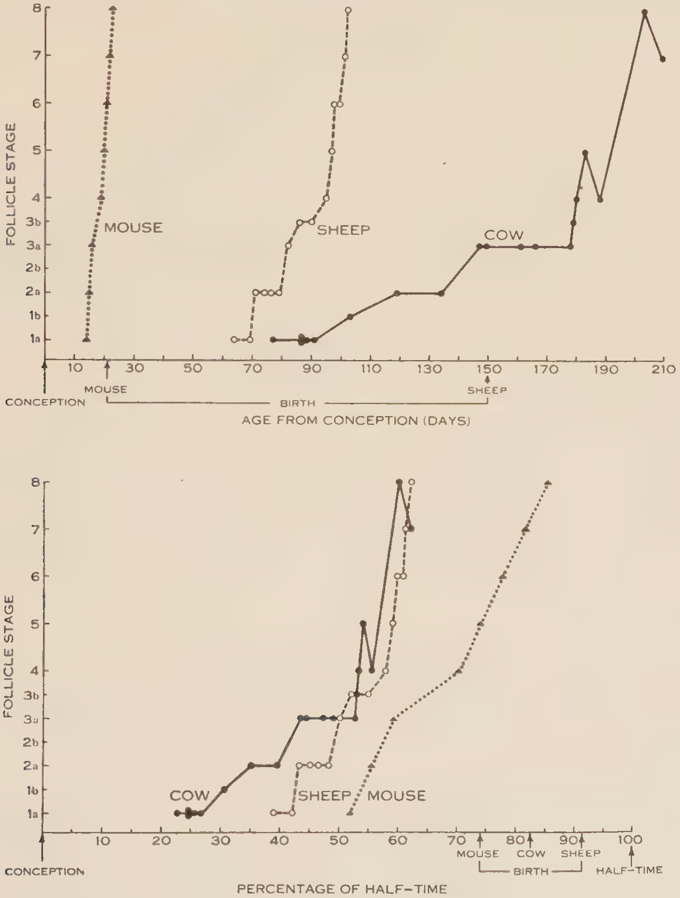


Fig. 9.—Relation between the age and the most advanced follicle stage (from 1–8) in *B. taurus*, sheep (Hardy and Lyne 1956), and mouse (Hardy 1949), in terms of the absolute time scale and an equivalent time scale (see text).

(*b*) *Follicle and Hair Density*

The density of follicles and hairs at various stages of development in the cow and sheep have also been compared using absolute and equivalent time scales (Fig. 10).

Note that the maximum follicle density in the cow is reached before, and in the sheep (Carter and Hardy 1947) after, the first hairs emerge. The maximum hair density in both the cow and sheep is reached during the late pre-natal period.

The percentage of medullation, as seen in the sections, is shown in Table 2. As all club hairs are non-medullated no medullation is seen in skin sections when all the follicles are at stage 10*c* (Table 2, birth samples (b)–(e)). Some of these hairs would, however, be medullated at a higher level.

V. Development of the Skin

The macroscopic calliper measurements of the thickness of the skin samples clearly show that the skin increases in total thickness throughout the foetal period, from the beginning of follicle initiation to birth (Fig. 8). Microscopic measurements of the thickness of the epidermis and dermis were made on vertical serial sections of the skin cut parallel to the long axes of the hair follicles.

Since clearly defined developmental stages of the hair follicles have been determined it has been possible to correlate some of the thickness changes which occur in the skin with those which take place in the follicles and sweat glands. Some of these changes are diagrammatically shown in Figure 8.

There is a well-defined increase and subsequent decrease in the thickness of the epidermis during the foetal period. During the period of follicle initiation and while the follicles are growing in length, the epidermis becomes approximately twice as thick as it was at the beginning of follicle initiation. The thicker epidermis contains a greater number of cell layers and a more prominent stratum spinosum.

The dermis (and total skin) increases in thickness throughout the entire period of foetal life. No attempt has been made to make separate measurements of the papillary and reticular layers described in adult cattle skin by Dowling (1955*b*) and Walker (1957*a*).

Because the angle of slope of the follicle is usually less than 90° its depth is less than its length. The maximum depth reached by the longest follicles measured is slightly more than half their maximum length. The maximum sweat gland depth is reached at birth whereas the maximum length is found at approximately 260 days gestation.

VI. Discussion

(*a*) *Rate of Follicle Development*

The rate of follicle development in the cow (*B. taurus*), sheep, and mouse has been compared in terms of the absolute time scale and an equivalent time scale (Fig. 9).

The first-formed follicles in the cow develop from stage 1 to 8 in approximately 126 days while the equivalent follicles in the sheep (Hardy and Lyne 1956) and mouse (Hardy 1949) pass through the same stages in about 38 and 9 days respectively. With the aid of the percentage "half-time" scale proposed by Lyne and Verhagen (1957) the rate of follicle development in the cow is compared with that in the sheep and mouse. This makes the mouse the slowest, while the cow and sheep have a similar rate of growth. The *half-time* is *the time taken to reach half the ultimate linear equivalence* (*or cube root of the body weight*) *when measured from conception to adulthood.*

The first hair cycle in the cow extends over a period of approximately 200 days compared with approximately 25 days in the mouse (Davidson and Hardy 1952).

Winters, Green, and Comstock (1942) observed the first appearance of hair follicles in cattle in a 90-day foetus, and the complete covering of the body with hairs at 230 days. Macroscopic observations on the emergence of hair were also included in Postma's (1947) criteria for age determination of cattle foetuses. He states that both 210- and 212-day foetuses may be covered with short hair. Both these studies thus support present observations which show follicles on the mid-lateral region of the trunk first appearing at about 77 days and hairs emerging at about 203 days.

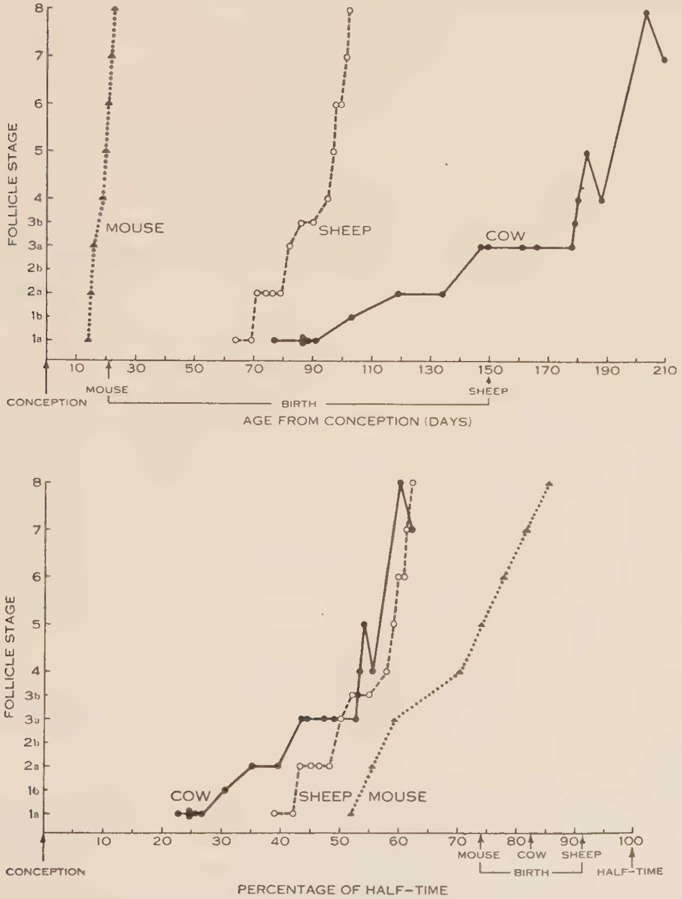


Fig. 9.—Relation between the age and the most advanced follicle stage (from 1–8) in *B. taurus*, sheep (Hardy and Lyne 1956), and mouse (Hardy 1949), in terms of the absolute time scale and an equivalent time scale (see text).

(*b*) *Follicle and Hair Density*

The density of follicles and hairs at various stages of development in the cow and sheep have also been compared using absolute and equivalent time scales (Fig. 10).

Note that the maximum follicle density in the cow is reached before, and in the sheep (Carter and Hardy 1947) after, the first hairs emerge. The maximum hair density in both the cow and sheep is reached during the late pre-natal period.

Another feature to be noted in this comparison is that in the sheep the maximum follicle density is reached after the maximum hair density whereas in the cow it occurs before this stage.

In Merino sheep, follicle initiation from the epidermis has ceased at about 115 days gestation (Lyne, unpublished data). The increasing follicle density thereafter can be explained by branching of the secondary follicles.

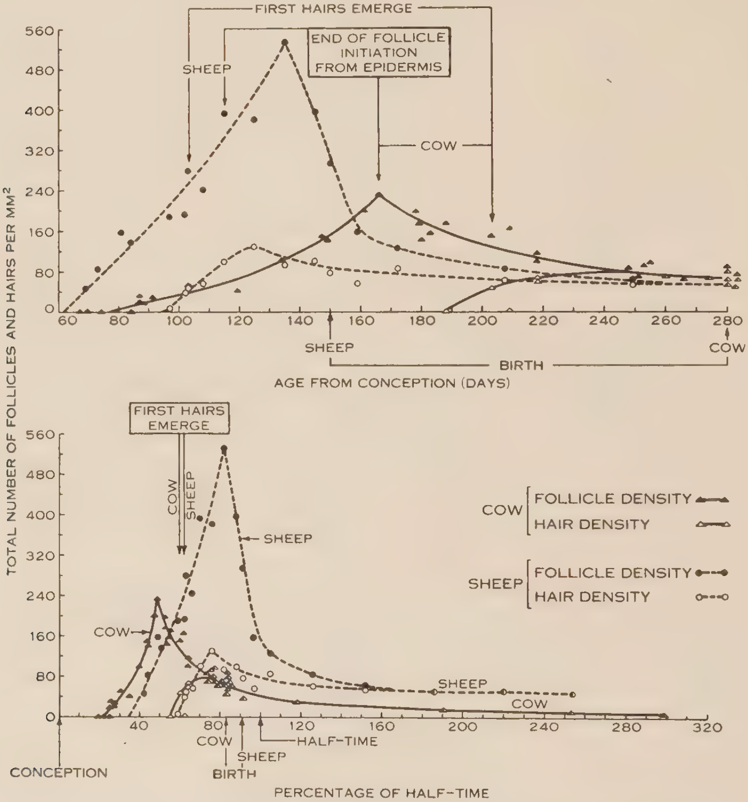


Fig. 10.—Relation between the age and the total density of the follicles and hairs in *B. taurus* and sheep (pre-natal, Carter and Hardy 1947; post-natal, Lyne, unpublished data), in terms of the absolute time scale and an equivalent time scale (see text). The post-natal data for cattle is taken from Carter and Dowling (1954). The curves through the points have been drawn by free-hand.

It is perhaps rather unexpected that the hair density in the new-born calf is even slightly greater than in Merino sheep at the same conception age (i.e. approximately 4 months after birth). In the adult cow, however, the hair density is very much less than at birth (Carter and Dowling 1954), whereas in the Merino sheep there is no marked post-natal change in the fibre density (Fig. 10, percentage of half-time scale) because of the maturation of secondary follicles accompanying skin expansion during growth. The cow, on the other hand, has all its follicles mature (i.e. beyond stage 6) at birth.

It is clearly seen that at the time when the first hairs emerge there is very little difference in equivalent age between the two species.

In foetuses of the Egyptian buffalo (*Bos* (*Bubalus*) *bubalis* L.), Hafez, Badreldin, and Shafei (1955*a*) found follicle densities (uncorrected for shrinkage) of 28·8, 105·6, and 12·5 per mm² on the fore-flank region at 1 month, 5 months, and birth respectively. The present authors wonder whether there could have been some error in the age determination of the 1-month old foetus for it seems most unlikely that hair follicles, if they are present at this early age, are still without hairs at 5 months.

(*c*) *Follicle Grouping*

In the cow there is an absence of clearly defined follicle groups, characteristic of sheep and other mammals. De Meijere (1894) states that rather indistinct groups can be seen. In the present study follicle groups are sometimes quite apparent below the sebaceous gland level (Plate 5, Fig. 4) but are indistinct at a higher level (Plate 5, Fig. 5). As in sheep (Hardy and Lyne 1956) most of the follicles formed after the trio group are situated on the ectal side of the group.

(*d*) *Paired Follicles*

Branching of follicles as found in sheep (Hardy and Lyne 1956), bandicoots (Lyne 1957*a*), and other mammals has not been observed in the cow. There is, however, the phenomenon of pairing, apparently not previously described in mammals, where two (occasionally three) follicles, arising independently from the epidermis, share a common hair canal. None of the pre- or post-natal studies of hair follicles in cattle refer to this pairing which is apparent in the present observations, particularly in advanced foetuses and new-born calves.

(*e*) *Ental Swelling*

The ental swelling, characteristic of the first-formed follicles in the cow, has been described in various mammals. In the cow this swelling is closely associated with the arrector pili muscle and a similar association is described in man by Stöhr (1903).

Stöhr considers various theories concerning the origin and function of the swelling. On the one hand there is the belief that it is the arrector pili muscle, attached to the outer root sheath and not lengthening at the same rate as the follicle, which pulls out its side to form the swelling. Against this opinion is the fact that the swelling is present even when there is little or no muscle, as in the guinea pig (Segall 1918) and the bandicoot (Lyne 1957*a*), at least in the first hair cycle. From present observations on the shape of the swelling and the manner in which the nuclei are arranged (Plate 1, Figs. 4, 5, and 6), it would seem that the swelling is not formed by traction of the muscle. Also, occasionally the swelling even extends around to the ectal side of the follicle. Thus its function perhaps is simply to provide a base to enable better attachment of the muscle to the follicle. Stöhr suggests, moreover, that during hair replacement, when the follicle bulb shrinks up, the swelling becomes a "Haarbeet" in which the club hair is held until it is shed. He also refers to other authors who hold that this "bed" is a possible source of future accessory hairs.

In cases where a swelling exists in the presence of little or no muscle, it may simply be a vestige of little functional significance.

(*f*) *Changes in the Skin in Relation to the Hair Growth Cycle*

The changes in the skin in relation to the first hair cycle in the cow are similar to those which have been described for hair cycles in the mouse (Chase, Montagna, and Malone 1953; Herrmann *et al.* 1955) and rat (Griesemer 1956). As the follicles develop the skin becomes much thicker, chiefly due to an increase in the thickness of the dermis. The increased thickness of the epidermis during the period of follicle activity in the cow (Fig. 8), is similar to that described in both the mouse and rat.

The skin must reach its adult thickness, at least in some breeds, early in post-natal life, as Dowling (1955*b*) has shown that "there is no significant difference between Australian Illawarra Shorthorn animals 6 months, 27 months, and 10 years old". Similar results are recorded by Walker (1957*a*). Patel and Anderson (1958), however, found a marked association of the skin thickness with age from birth to 2 years.

(*g*) *Conclusions*

The studies reported in this paper are a necessary preliminary step to further pre- and post-natal histological investigations of the skin, hair follicles, and associated glands in different breeds of cattle. At the present time there is widespread interest in various aspects of the adaptation of cattle to tropical environments. Post-natal studies of the skin, with particular emphasis on seasonal and nutritional influences and the changes in relation to the hair growth cycle should be fruitful.

A comparison of the findings reported in the present study with the pre-natal development of skin and hair in Zebu crossbreds (*B. indicus* × *B. taurus*) will be dealt with in a subsequent paper.

VII. ACKNOWLEDGMENTS

The authors are particularly indebted to Mr. R. H. Hayman, Officer-in-Charge, F. D. McMaster Field Station, Division of Animal Health and Production, C.S.I.R.O., for the new-born material, and to Mr. P. Bryden, State Abattoir, Homebush, N.S.W., for 25 of the foetuses. Prof. K. W. Cleland, University of Sydney, and Dr. D. F. Dowling and Mr. P. G. Schinckel, Division of Animal Health and Production, C.S.I.R.O., kindly read the manuscript. Also, we wish to thank Miss A. J. Bathgate and Mr. E. W. Taylor for technical assistance, Mr. T. Dagg for photography, and Mr. J. C. Coles and Miss B. Miller for preparing Figures 4–10 for publication.

VIII. REFERENCES

BARISSON VILLARES, J., and BERTHET, L. A. (1951*a*).—Contribuição para o estudo do aparelho pilo-sebáceo-músculo-sudoríparo nos *Bos taurus* e *Bos indicus*. *Bol. Industr. Anim.* **12**: 3–20.

BARISSON VILLARES, J., and BERTHET, L. A. (1951*b*).—Contribuição para o estudo da estrutura da glândula sudorípara nos *Bos taurus* e *Bos indicus*. *Bol. Industr. Anim.* **12**: 21–44.

BONSMA, J. C. (1949).—Breeding cattle for increased adaptability to tropical and subtropical environments. *J. Agric. Sci.* **39**: 204–21.

Bonsma, J. C., and Pretorius, A. J. (1943).—Influence of colour and coat cover on adaptability of cattle. *Fmg. in S. Afr.* **18**: 101–20.

Brody, S. (1945).—"Bioenergetics and Growth." (Reinhold Publ. Co.: New York.)

Camek, J. (1920).—Investigations of the hair of different breeds of cattle. *J. Agric. Sci.* **10**: 12–21.

Carter, H. B., and Dowling, D. F. (1954).—The hair follicle and apocrine gland population of cattle skin. *Aust. J. Agric. Res.* **5**: 745–54.

Carter, H. B., and Hardy, Margaret H. (1947).—Studies in the biology of the skin and fleece of sheep. 4. The hair follicle group and its topographical variations in the skin of the Merino foetus. Bull. Coun. Sci. Industr. Res. Aust. No. 215.

Chase, H. B., Montagna, W., and Malone, Janet D. (1953).—Changes in the skin in relation to the hair growth cycle. *Anat. Rec.* **116**: 75–82.

Davidson, Pamela, and Hardy, Margaret H. (1952).—The development of mouse vibrissae *in vivo* and *in vitro*. *J. Anat., Lond.* **86**: 342–56.

Dowling, D. F. (1955*a*).—The hair follicle and apocrine gland populations of Zebu (*Bos indicus* L.) and Shorthorn (*B. taurus* L.) cattle skin. *Aust. J. Agric. Res.* **6**: 645–54.

Dowling, D. F. (1955*b*).—The thickness of cattle skin. *Aust. J. Agric. Res.* **6**: 776–85.

Duerden, J. E., and Whitnall, A. B. M. (1930).—Seasonal variation in the coat of some domestic mammals. *S. Afr. J. Sci.* **27**: 521–45.

Ferguson, K. A., and Dowling, D. F. (1955).—The function of cattle sweat glands. *Aust. J. Agric. Res.* **6**: 640–4.

Findlay, J. D., and Yang, S. H. (1948).—Capillary distribution in cow skin. *Nature* **161**: 1012–3.

Findlay, J. D., and Yang, S. H. (1950).—The sweat glands of Ayrshire cattle. *J. Agric. Sci.* **40**: 126–33.

French, M. H. (1946).—Growth rates of hair on grade European and indigenous breeds of cattle. *E. Afr. Agric. J.* **11**: 181–3.

Goodall, A. Myfanwy, and Yang, S. H. (1952).—Myoepithelial cells in bovine sweat glands. *J. Agric. Sci.* **42**: 159–61.

Goodall, A. Myfanwy, and Yang, S. H. (1954).—The vascular supply of the skin of Ayrshire calves and embryos. *J. Agric. Sci.* **44**: 1–4.

Griesemer, R. D. (1956).—Change in enzyme activity and ribonucleic acid concentration within the epidermal cell of the rat during the growth stage of the hair cycle. *J. Biophys. Biochem. Cytol.* **2**: 523–30.

Hafez, E. S. E., Badreldin, A. L., and Shafei, M. M. (1955*a*).—The hair coat in Bovinae. *Emp. J. Exp. Agric.* **23**: 34–8.

Hafez, E. S. E., Badreldin, A. L., and Shafei, M. M. (1955*b*).—Skin structure of Egyptian buffaloes and cattle with particular reference to sweat glands. *J. Agric. Sci.* **46**: 19–30.

Hardy, Margaret H. (1949).—The development of mouse hair *in vitro* with some observations on pigmentation. *J. Anat., Lond.* **83**: 364–84.

Hardy, Margaret H., and Lyne, A. G. (1956).—The pre-natal development of wool follicles in Merino sheep. *Aust. J. Biol. Sci.* **9**: 423–41.

Herrmann, F., Morrill, S. D., Sherwin, R. W., Rothstein, M. J., and Sulzberger, Marion B. (1955).—Factors influencing the incidence of epidermal methylcholanthrene tumors in mice treated with cortisone. III. Studies of the hair follicular cycle ("skin cycle") in relation to the incidence of tumors after cortisone administration. *J. Invest. Derm.* **25**: 423–38.

Lyne, A. G. (1957*a*).—The development and replacement of pelage hairs in the bandicoot *Perameles nasuta* Geoffroy (Marsupialia: Peramelidae). *Aust. J. Biol. Sci.* **10**: 197–216.

Lyne, A. G. (1957*b*).—The development of the epidermis and hair canals in the Merino sheep foetus. *Aust. J. Biol. Sci.* **10**: 390–7.

Lyne, A. G., and Verhagen, A. M. W. (1957).—Growth of the marsupial *Trichosurus vulpecula* and a comparison with some higher mammals. *Growth* **21**: 167–95.

de Meijere, J. C. H. (1894).—Über die Haare der Säugethiere, besonders über ihre Anordnung. *Morph. Jb.* **21**: 312–424.

Nay, T., and Dowling, D. F. (1957).—Size of sweat glands in Shorthorn strains and Zebu × Shorthorn crossbred cattle. *Aust. J. Agric. Res.* **8**: 385–93.

Nay, T., and Hayman, R. H. (1956).—Sweat glands in Zebu (*Bos indicus* L.) and European (*B. taurus* L.) cattle. I. Size of individual glands, the denseness of their population, and their depth below the skin surface. *Aust. J. Agric. Res.* **7**: 482–94

Patel, U. G., and Anderson, D. W. (1958).—Variation of skin thickness in dairy cattle. *Emp. J. Exp. Agric.* **26**: 18–24.

Postma, C. (1947).—Die Ouderdomsbepaling bij Runder Foetus. *Tijdschr. Diergeneesk.* **72**: 463–531.

Segall, A. (1918).—Ueber die Entwicklung und den Wechsel der Haare beim Meerschweinchen (*Cavia cobaya* Schreb). *Arch. Mikr. Anat.* **91**: 218–91.

Stöhr, Ph. (1903).—Entwickelungsgeschichte des menschlichen Wollhaares. *Anat. Hefte* **23**: 1–66.

Walker, C. A. (1957*a*).—The skin thickness of cattle in Northern Rhodesia. *J. Agric. Sci.* **49**: 211–3.

Walker, C. A. (1957*b*).—Studies of the cattle of Northern Rhodesia. II. The apocrine gland population of the skin of Northern Rhodesian cattle and its connexion with the heat toleration coefficient. *J. Agric. Sci.* **49**: 401–4.

Winters, L. M., Green, W. W., and Comstock, R. E. (1942).—Prenatal development of the bovine. Bull. Minn. Agric. Exp. Sta. No. 151.

Yamane, J., and Ono, Y. (1936).—Rassenanatomische Untersuchungen der Hautstruktur vom Büffel, Zebu, Formosarind und Friesisch-Holländer im Hinblick auf das Problem der Tropenanpassung. *Mem. Fac. Sci. Agric. Taihoku* **19**: 87–136.

Yang, S. H. (1952).—Histochemical studies on bovine sweat glands. *J. Agric. Sci.* **42**: 155–8.

Yeates, N. T. M. (1954).—Environmental control of coat changes in cattle. *Nature* **174**: 609–10.

Yeates, N. T. M. (1955).—Photoperiodicity in cattle. I. Seasonal changes in coat character and their importance in heat regulation. *Aust. J. Agric. Res.* **6**: 891–902.

Yeates, N. T. M. (1958).—Observations on the role of nutrition in coat shedding in cattle. *J. Agric. Sci.* **50**: 110–2.

Explanation of Plates 1–5

A, arrector pili muscle; *C*, stratum corneum; *CH*, club hair; *CHC*, common epidermal hair canal; *Co*, cortex; *CTS*, connective tissue sheath; *CuH*, cuticle of hair; *CuI*, cuticle of inner root sheath; *D*, dermis; *DP*, dermal papilla; *E*, epidermis; *ES*, ental swelling of outer root sheath; *FF*, first-formed follicle; *G*, stratum germinativum; *Gr*, cells containing keratohyalin granules; *H*, keratinized hair; *HC*, hair canal; *He*, Henle's layer; *HG*, hair germ; *HN*, hair cone; *Hu*, Huxley's layer; *I*, inner root sheath; *K*, keratinized cells of hair canal; *L*, lumen of sweat gland; *LsF*, last-formed follicle; *LtF*, later-formed follicle; *M*, medulla; *O*, outer root sheath; *PF*, paired follicles; *PP*, pre-papilla; *RP*, resting papilla; *S*, stratum spinosum; *SCS*, space formed by disintegration of sebaceous cells; *SG*, sebaceous gland with differentiated cells; *SwG*, sweat gland; *SwGD*, sweat gland duct; *SwGR*, sweat gland rudiment; *T*, trio group

Plate 1

Longitudinal sections of first-formed follicles showing their development

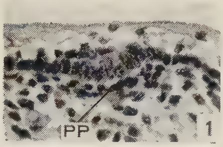
Fig. 1.—Stage 1*a* in an 88-day foetus.

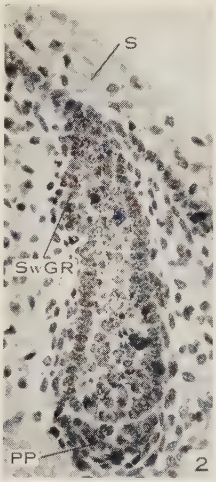
Fig. 2.—Early stage 2*a* in a 119-day foetus.

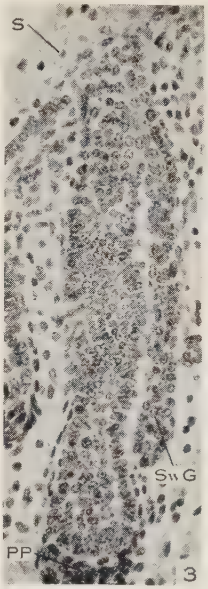
Fig. 3.—Late stage 2*a* in a 134-day foetus.

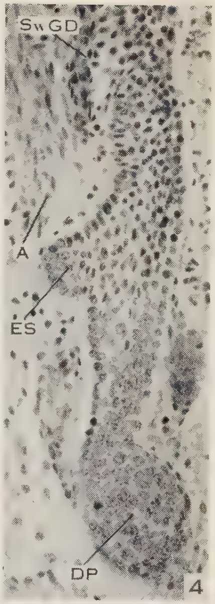
Fig. 4.—Stage 3*a* in a 179-day foetus. Note the prominent ental swelling.

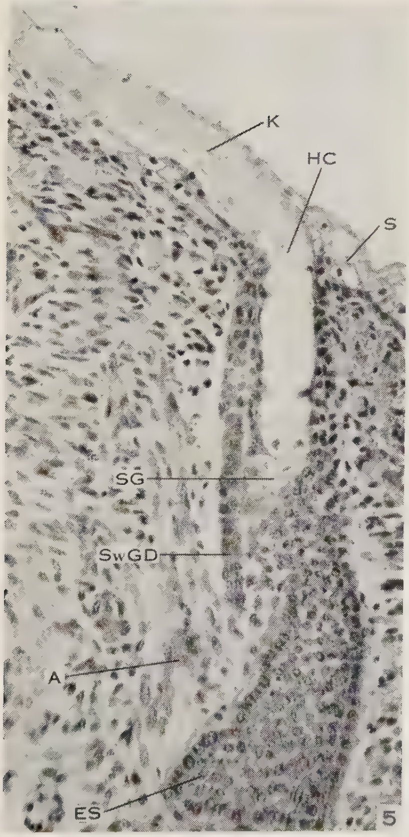
SKIN AND HAIR DEVELOPMENT IN CATTLE



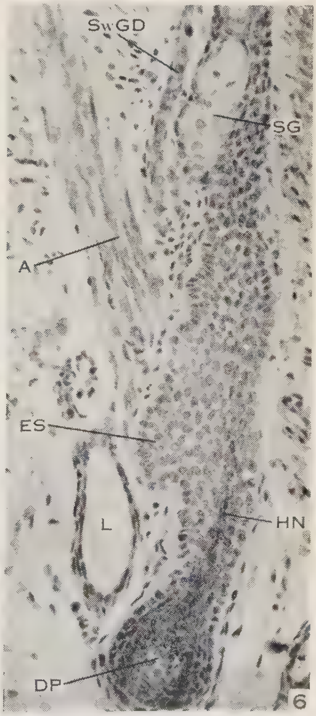




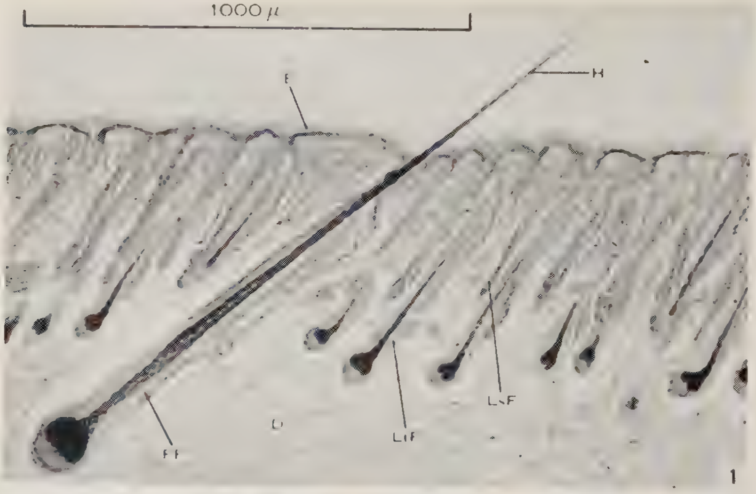


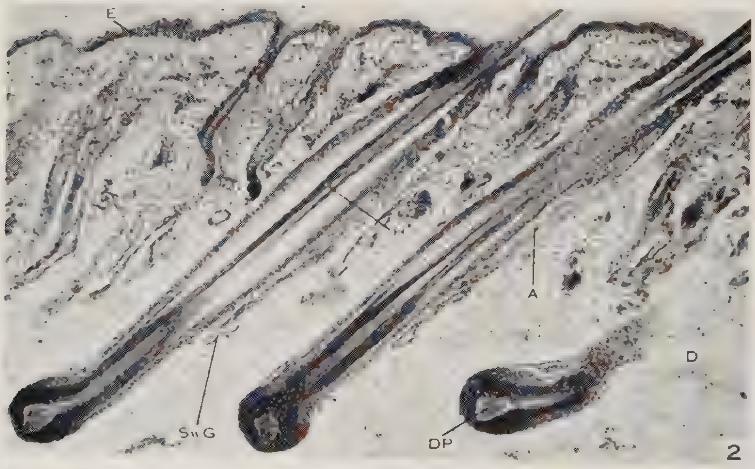


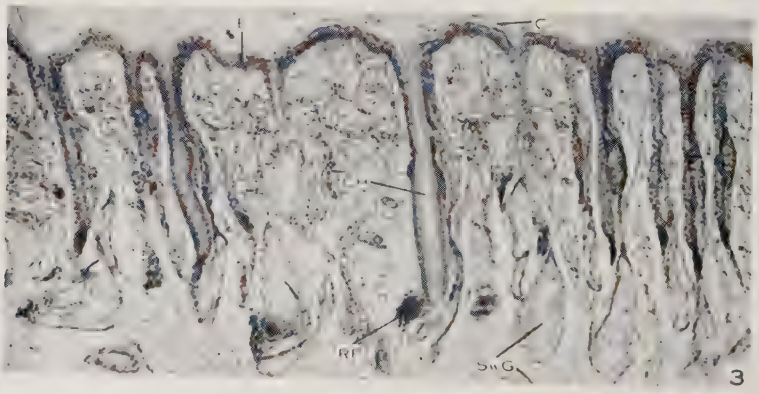
100 μ

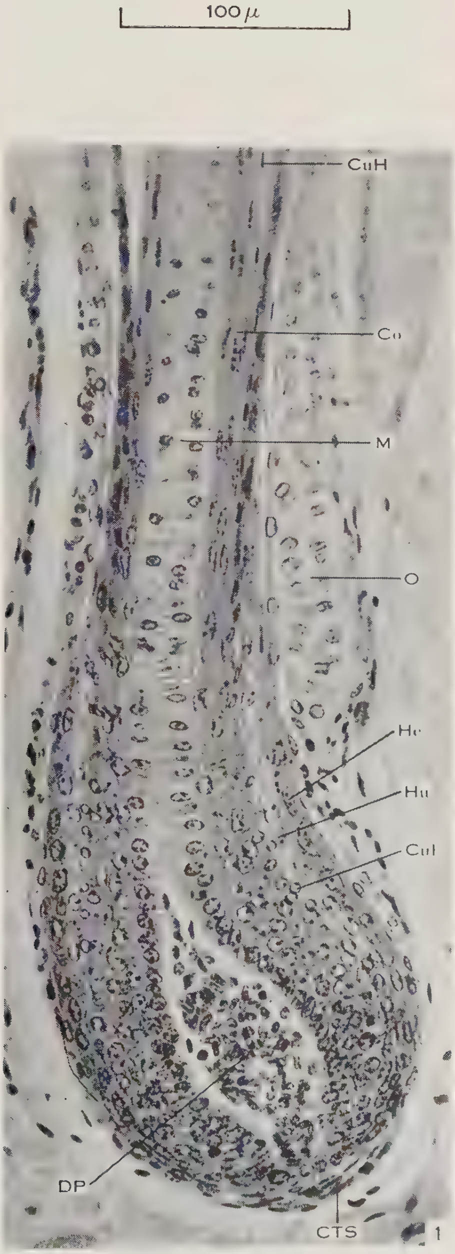


SKIN AND HAIR DEVELOPMENT IN CATTLE

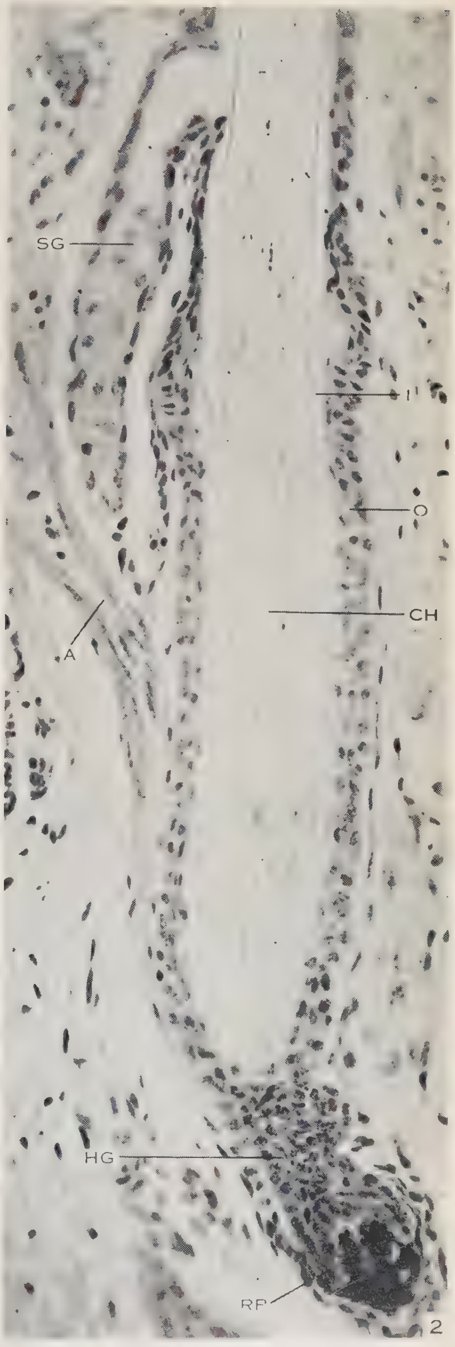






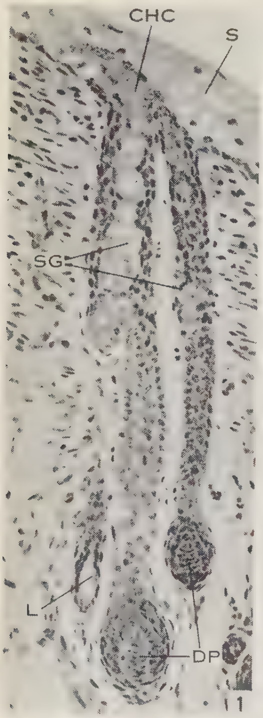


1

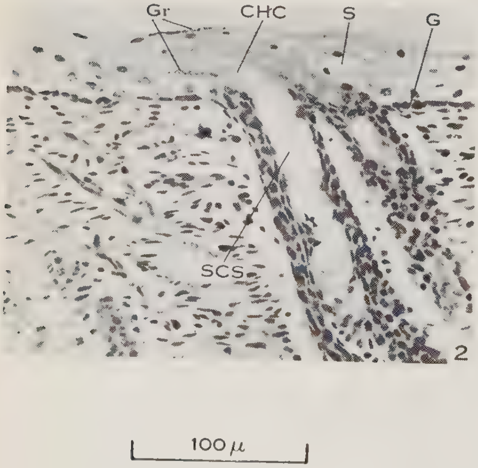


2

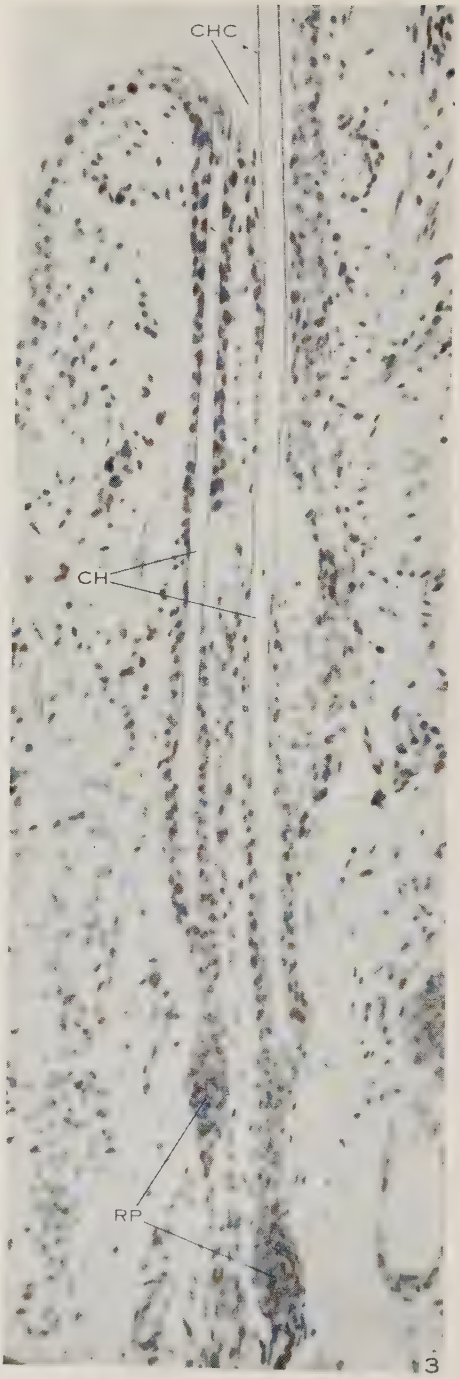
SKIN AND HAIR DEVELOPMENT IN CATTLE



1

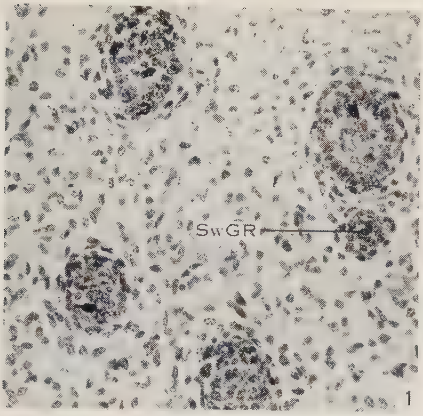


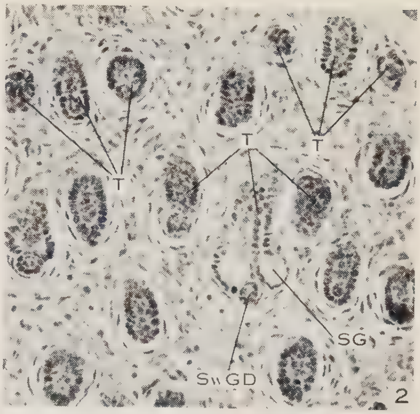
2

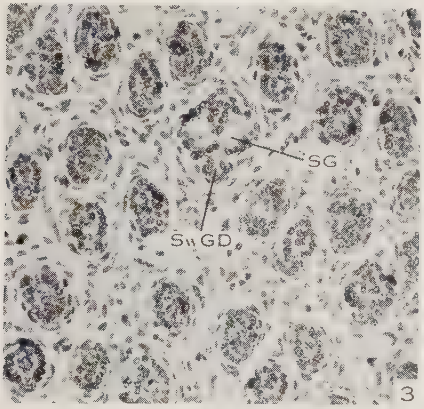


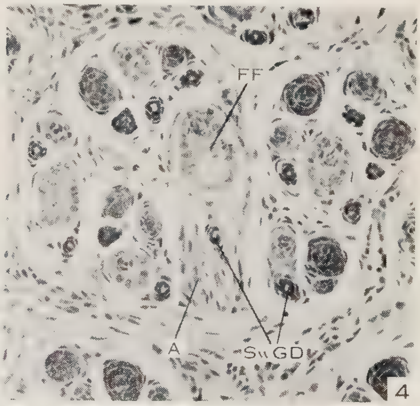
3

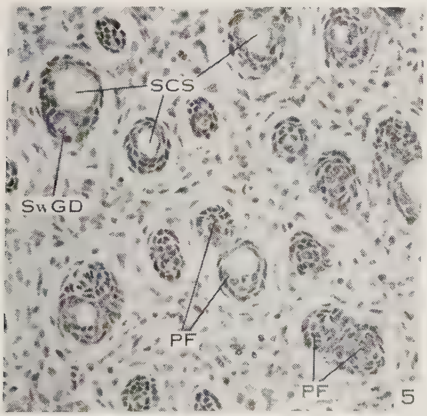
SKIN AND HAIR DEVELOPMENT IN CATTLE

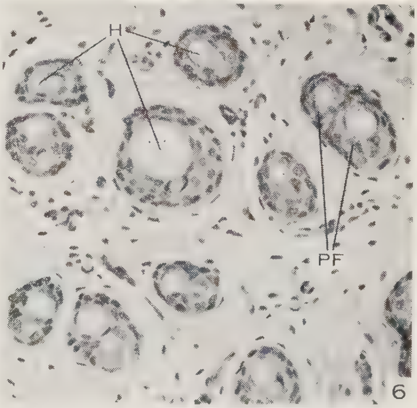












100 μ

Fig. 5.—Stage 3*b* in a 179-day foetus showing the prominent ental swelling and developing hair canal.

Fig. 6.—Stage 4 in a 183-day foetus.

PLATE 2

Development of the first-formed follicles

Fig. 1.—Vertical section of skin from a 203-day foetus showing a first-formed follicle at stage 8. Later- and last-formed follicles are at stages 3*b*–7.

Fig. 2.—Longitudinal section of two first-formed follicles at stage 9 in a new-born calf (Australian Illawarra Shorthorn × Shorthorn).

Fig. 3.—Vertical section of skin from a new-born calf (Jersey) with all follicles at stage 10*c*.

PLATE 3

Development of the first-formed follicles

Fig. 1.—Longitudinal section of the lower part of an active follicle (stage 9) in a 251-day foetus.

Fig. 2.—Longitudinal section of the lower part of a quiescent follicle (stage 10*c*) in a new-born calf.

PLATE 4

Paired follicles

Fig. 1.—Longitudinal section of paired follicles at stage 3*b* in a 183-day foetus.

Fig. 2.—Longitudinal section of the upper part of paired follicles at stage 3*b* in a 183-day foetus, showing the common hair canal at a slightly later stage of development than in Plate 4, Figure 1.

Fig. 3.—Longitudinal section of paired follicles at stage 10*c* in a 251-day foetus.

PLATE 5

Transverse sections showing development of the follicle population

Fig. 1.—Most advanced of first-formed follicles at stage 2*a* in a 119-day foetus.

Fig. 2.—Trio groups formed by the development of later-formed follicles on either side of the first-formed follicles in a 147-day foetus.

Fig. 3.—Follicles at the stage when the density is at a maximum (233 per mm^2) in a 166-day foetus.

Fig. 4.—Follicles at the level of the ental swelling showing the appearance of a group in a 183-day foetus. Most advanced follicles at stage 5 and least advanced at stage 3*a*.

Fig. 5.—The same specimen as in Plate 5, Figure 4, showing some paired follicles at a superficial level.

Fig. 6.—Follicles at a superficial level in a new-born calf, showing paired follicles.

THE ELECTROPHORETIC PROPERTIES OF *S*-CARBOXYMETHYLKERATEINES AND *S*-SULPHOKERATEINES FROM WOOL

By E. F. Woods*

[*Manuscript received August* 21, 1958]

Summary

The most suitable conditions for the moving-boundary electrophoresis of *S*-carboxymethylkerateines were determined. The use of urea or sodium dodecyl sulphate to assist the extraction of soluble proteins from wool with alkaline thioglycollate did not alter the electrophoretic behaviour of the extracted proteins.

S-sulphokerateines, prepared from wool by a new method due to Swan (1957), exhibited similar behaviour to the carboxymethylkerateines on electrophoresis and this method of preparation can be adapted to fractional extraction to give one component more homogeneous electrophoretically than the remainder.

Proteins prepared by fractional extraction of wool with 8M urea and sodium bisulphite at pH 5 gave similar results on electrophoretic examination to those obtained by fractional extraction with thioglycollate at pH 11.

The effects of protein interactions, including aggregation, on the electrophoresis diagrams of soluble wool proteins are discussed.

I. Introduction

Keratin derivatives which are water soluble and stabilized against re-oxidation can be readily prepared from wool by alkaline reduction of the disulphide bonds and substitution of the thiol groups so produced (Goddard and Michaelis 1935; Gillespie 1956). Reagents such as thioglycollic acid, mercaptoethanol, and cysteine give two thiol groups per cystine residue. Sodium bisulphite (or sulphite), which has been widely used in the presence of concentrated solutions of urea, guanidine, and anionic detergents to prepare soluble keratins, converts one of the cystine sulphur atoms to $-SSO_3^-$, the other to $-SH$. Such an unsymmetrical fission must complicate the examination of the soluble proteins for heterogeneity by conventional means. Swan (1957) has shown in this Laboratory that the disulphide bonds of wool can be split specifically and symmetrically by means of sodium sulphite in the presence of cupric ions whereby water-soluble *S*-sulphokerateines may be obtained. In this paper the *S*-sulphokerateines have been examined by moving-boundary electrophoresis and compared with the *S*-carboxymethylkerateines prepared from wool by the alkaline thioglycollate reduction method of Gillespie and Lennox (1953, 1955*a*) and some modifications of it.

II. Experimental

(*a*) *Preparation of* S-*Carboxymethylkerateines*

The kerateines were prepared from Merino 64's solvent-scoured wool top by the method of Gillespie and Lennox (1953) using a single extraction at 50°C for

---

*Divison of Protein Chemistry (formerly Biochemistry Unit), C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

20 min with 0·1M sodium thioglycollate at pH 12·3 whereby approximately 60 per cent. of the wool dissolved. A liquor to wool ratio of 100 ml of thioglycollate solution to 1 g wool was used. After extraction the pH was lowered to approximately 8·5 and the –SH groups allowed to react with sodium iodoacetate, the pH being kept constant by the addition of alkali. When the nitroprusside reaction was negative the solution was dialysed against tap-water for 48 hr and concentrated by pervaporation; alternatively the proteins could be first precipitated by half saturation with ammonium sulphate and the precipitates dialysed to remove ammonium sulphate. Several modifications of the extraction procedure were examined. In the presence of 0·5 per cent. sodium dodecyl sulphate or 8M urea, sodium thioglycollate at pH 10·5–11·0 dissolved the same amount of wool as at the higher pH and temperature.

The action of sodium dodecyl sulphate and urea in assisting the solution of the wool proteins is probably due to the fact that both these reagents are excellent disaggregating agents for the isolated proteins (O'Donnell and Woods 1956*a*), and it has also been found that the disulphide bonds of proteins are more readily reduced in the presence of either urea or sodium dodecyl sulphate (Lindley 1955; Markus and Karush 1957).

(*b*) *Preparation of* S-*Sulphokerateines*

The *S*-sulphokerateines were prepared by the method described by Swan (1957) who used the following reactions of cupric ions in the presence of sulphite for the conversion of the disulphide and thiol groups of proteins to $-SSO_3^-$:

$$\mathrm{RSSR}+2\mathrm{Cu}^{2+}+2\mathrm{SO}_3^{2-}\longrightarrow 2\mathrm{RSSO}_3^{-}+2\mathrm{Cu}^{+} \quad \ldots\ldots(1)$$

$$\mathrm{RSH}+2\mathrm{Cu}^{2+}+\mathrm{SO}_3^{2-}\longrightarrow \mathrm{RSSO}_3^{-}+2\mathrm{Cu}^{+}+\mathrm{H}^{+} \quad \ldots\ldots(2)$$

With this method in the presence of 8M urea, 70 per cent. of wool could be dissolved overnight and up to 85 per cent. with longer extraction times. If the urea is omitted the extraction process is slower but the same amount of protein dissolves after about 8 days. The protein solubilities found by this method were in substantial agreement with the values given by Swan (1957).

For electrophoresis the protein was prepared by a fractional extraction process at 2°C. The reagent was 0·04M in cupric ammonium hydroxide, prepared by the addition of conc. NH_4OH to $CuSO_4$, 0·1M in Na_2SO_3, and was adjusted to approximately pH 10 with conc. NH_4OH. A liquor to wool ratio of 100 : 1 was used. Forty per cent. of the wool had dissolved after 18 hr, the solution was filtered and the residue extracted with fresh reagent for 48 hr. A further 25 per cent. of protein went into solution. Triammonium citrate was then added to the extracts (more than sufficient to complex with all the copper present), the pH lowered to about 7 by the addition of conc. HCl and the protein precipitated by the addition of ammonium sulphate to approximately two-thirds saturation. Acid precipitation recovered only 75 per cent. of the soluble proteins; ammonium sulphate precipitation was therefore preferred. The protein precipitates were dissolved in 0·1M potassium bicarbonate or 0·05M borax, more ammonium citrate was added, and the solutions dialysed against running tap-water. If all the copper was not removed as evidenced by a purple tint on making the protein solutions slightly alkaline, disodium ethylenediaminetetra-acetate was added and the solution redialysed.

Fractional extractions of wool were also made with urea bisulphite at pH 5·0. Under these conditions only one of the disulphide sulphur atoms is converted to $-SSO_3^-$, the other remaining as –SH. Reaction (2) can then be used to convert this –SH group to $-SSO_3^-$ after extraction. The conditions of extraction were similar to those used by Woods (1952) and Friend and O'Donnell (1953). 50 g wool was extracted with 300 ml of 8M urea and 0·04M $NaHSO_3$ at pH 5·0 for 48 hr at 50°C; 15 per cent. of the protein dissolved and a further 25 per cent. was extracted in 72 hr after the addition of fresh reagent. These extracts were converted to *S*-sulphokerateines by adjusting the pH to 10 with conc. ammonium hydroxide, adding cuprammonium hydroxide and more Na_2SO_3. After allowing a few minutes for the reaction to be completed, the solution was treated as above to recover the proteins. A further 25 per cent. of soluble protein was recovered from the residue by extraction with the cuprammonium sulphite at pH 10 for 24 hr at room temperature.

(*c*) *Cystine Analysis on Extracted Proteins*

Determinations of residual disulphide on the extracted proteins were done by Dr. S. J. Leach in this Laboratory. The intact proteins were titrated with mercuric chloride amperometrically at the dropping-mercury electrode in the presence of 0·2M sulphite and 8M urea at pH 9·2. All the *S*-carboxymethylkerateine preparations were free of disulphide, while of four *S*-sulphokerateine preparations one contained approximately 0·5 per cent. disulphide (expressed as cystine), the others contained none. The above method using the intact protein must be used in the case of the sulphokerateines since the $-SSO_3^-$ group is known to revert to –SH on acid hydrolysis.

(*d*) *Electrophoresis*

Electrophoresis of the wool proteins was carried out at 1°C in a standard moving-boundary Tiselius electrophoresis apparatus (LKB, Sweden), using the inclined-slit schlieren optical system for observation of the boundaries. The proteins were dialysed at 2°C for 48 hr against two changes of buffer before electrophoresis. The protein concentration was measured refractometrically after dialysis and adjusted to approximately 1·2 per cent. for electrophoresis. Mobilities were calculated using the conductivity of the protein solution.

III. RESULTS

(*a*) S-*Carboxymethylkerateines*

Figure 1 shows the ascending and descending patterns at various pH values of the combined unfractionated *S*-carboxymethylkerateines from wool, prepared by urea–thioglycollate (pH 11·0) extraction. The descending pattern is complex at pH values of 8·6 and 9·6, and as the pH is raised the proportion of components changes. In sodium carbonate buffer at pH 11 the areas under the peaks were different to those obtained with glycine buffer at this pH. With piperidine hydrochloride at pH 11 a slow-moving boundary, not evident in the other buffers, occurred. The most suitable buffer for electrophoresis was glycine–sodium hydroxide at pH 11 where there was the least difference between the mobilities of the ascending and descending peaks and the patterns from the two sides of the electrophoresis tube more nearly corresponded.

Figure 2 gives the patterns at pH 11 of the *S*-carboxymethylkerateines extracted with alkaline thioglycollate (pH 10·9) containing sodium dodecyl sulphate,

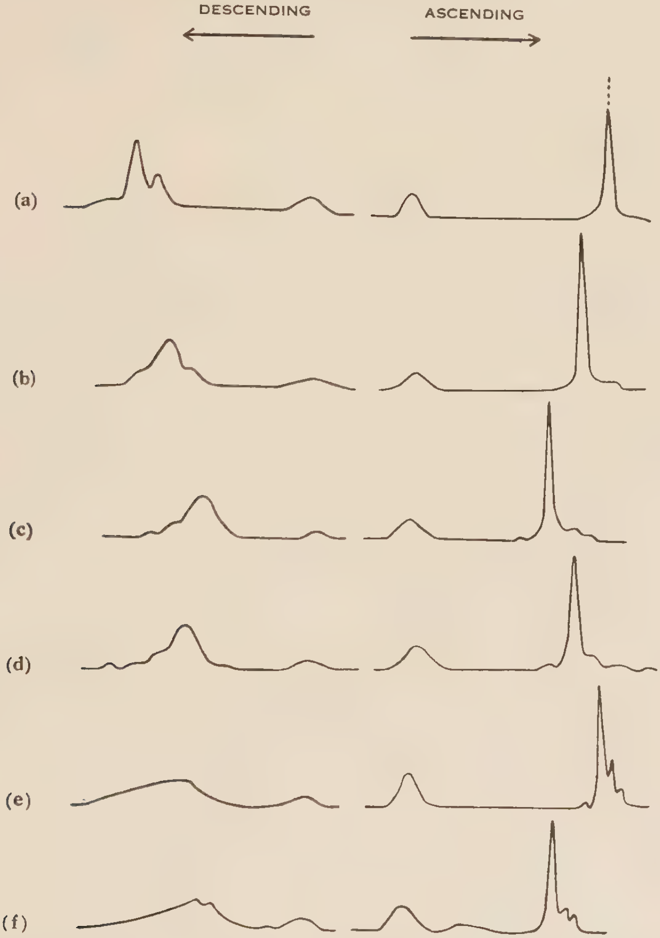


Fig. 1.—Electrophoresis in buffers of ionic strength 0·1 of *S*-carboxymethylkerateines from wool prepared by extraction at pH 11·0 with alkaline thioglycollate–8M urea. (*a*) Veronal, pH 8·6; (*b*) glycine, pH 9·6; (*c*) glycine pH 10·5; (*d*) glycine, pH 11·0; (*e*) sodium carbonate, pH 11·0; (*f*) piperidine hydrochloride, pH 10·9.

and by the Gillespie and Lennox (1953) alkaline thioglycollate procedure. The ascending patterns are almost identical with one another and with the previous urea–thioglycollate preparation (Fig. 1(*d*)). The splitting of the main peak on the descending side for the detergent alkaline thioglycollate method (Fig. 2(*b*)) was, however, not a reproducible property and has occurred with carboxymethylkerateines prepared by all three procedures. There is evidence that slight variations in the substitution of the protein –SH groups with iodoacetate may be important in determining anomalies in the pattern. In the preparation of the main component, *S*-carboxymethylkerateine 2 (SCMK2), by the fractional extraction procedure of Gillespie and Lennox (1955*a*) it has been observed that variations in the conditions for substitution with iodoacetate and subsequent dialysis sometimes led to double peaks

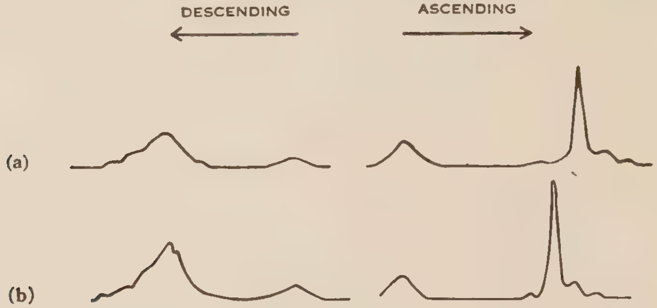


Fig. 2.—Electrophoresis in glycine buffer of ionic strength 0·1 and pH 11·0 of *S*-carboxymethylkerateines (*a*) prepared by alkaline thioglycollate extraction at pH 12·3, and (*b*) prepared by alkaline thioglycollate extraction at pH 11·0 in the presence of sodium dodecyl sulphate.

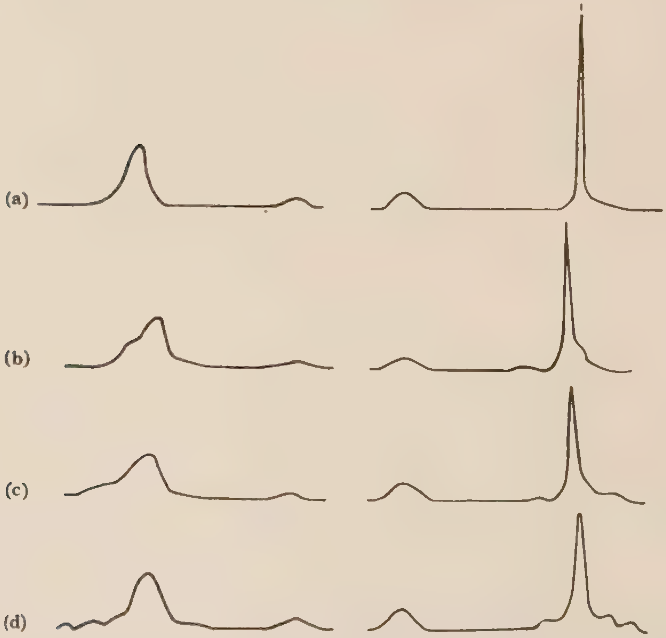


Fig. 3.—Electrophoresis in buffers of ionic strength 0·1 of *S*-sulphokerateines from wool. The proteins comprise the first 40 per cent. of extractable material. (*a*) Veronal, pH 8·6; (*b*) glycine, pH 9·5; (*c*) glycine, pH 10·6; (*d*) glycine, pH 11·1.

on the descending boundary. For example, when dialysis was carried out at pH 8 to remove the excess iodoacetate and dialysable products, a single peak was obtained on electrophoresis at pH 11, whereas lowering the pH to 6 after substitution followed by dialysis gave a double peak. Further, a preparation of SCMK2, which showed about 90 per cent. of the material as a single descending peak on electrophoresis of a 1·2 per cent. solution, gave a double peak on electrophoresis after it had been fractionated by the ammonium sulphate procedure of Gillespie (1957). At a protein concentration of 0·4 per cent., however, electrophoresis gave a single symmetrical peak in both limbs of the electrophoresis tube for the ammonium sulphate-fractionated protein.

(*b*) S-*Sulphokerateines*

Figures 3 and 4 show the electrophoretic patterns at four pH values of the *S*-sulphokerateines prepared by fractional extraction of wool with the cuprammonium sulphite reagent at pH 10. The first extract was obtained by extraction for 24 hr at 2°C and the second by a further extraction for 48 hr. The patterns for the first extract are similar to those observed for the *S*-carboxymethylkerateines and the best resolution of components occurs on electrophoresis at pH 11. The second extract is slightly more homogeneous and appears to correspond to the kerateine 2 of Gillespie and Lennox (1955*a*). In the combined extracts of the *S*-sulphokerateines it appears in the same position in the electrophoretic diagram as the kerateine 2 in the combined kerateine extracts, or the SCMK2 in the combined carboxymethyl proteins. For this reason it is labelled *S*-sulphokerateine 2 (SSK2) although there is some evidence that the protein fractionated from the early extracts of wool may not be the same as the SCMK2 of later extracts (Gillespie 1958). The electrophoretic pattern at low ionic strength (0·02) at pH 9·6 shows the descending pattern to be very spread out (Fig. 4(*e*)).

Figure 5 gives the electrophoretic patterns at pH 11 of the proteins obtained by the fractional extraction of wool with urea bisulphite at pH 5 and subsequent conversion to the *S*-sulphokerateines. The first extract represented 15 per cent. of the wool, the second 20 per cent., and the third extract, which was obtained by treating the residue with cuprammonium sulphite at pH 10, about 20 per cent.

IV. DISCUSSION

It is clear from Figures 1, 3, and 4 that the best resolution of the wool proteins is obtained on electrophoresis at pH 11 in 0·1 ionic strength glycine buffer. Fewer components are observed at lower pH values. At pH 11 glycine is at the upper limit of its alkaline buffering range but it was the most suitable buffer tried at this pH since it gave ascending and descending patterns which more nearly corresponded. The small fast-moving peak in the descending pattern, which moves faster than any peak on the ascending side, is possibly a false boundary of the type described by Svensson (1946) or Woods (1958). The electrophoretic patterns of the carboxymethylkerateines are similar to those obtained by Gillespie and Lennox (1953, 1955*a*) for the kerateines. Four components were resolved with possibly additional minor components. Conversion to the *S*-carboxymethyl derivatives is advantageous in that it

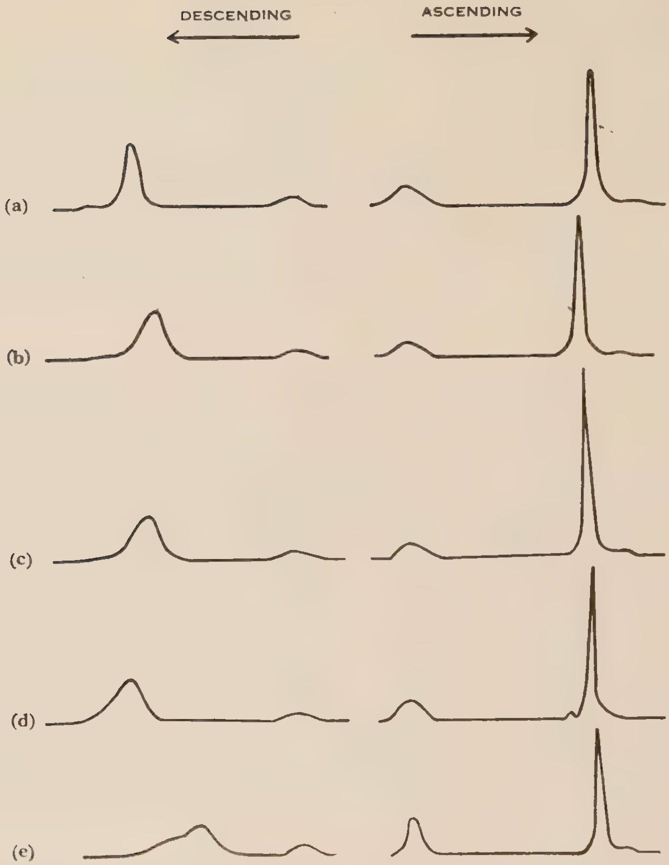


Fig. 4.—Electrophoresis in buffers of ionic strength 0·1 of *S*-sulphokerateines from wool. The protein represents the fraction labelled *S*-sulphokerateine 2, and comprises 20 per cent. of material from the second extraction of the wool. (*a*) Veronal, pH 8·6; (*b*) glycine, pH 9·6; (*c*) glycine, pH 10·5; (*d*) glycine, pH 11·1; (*e*) glycine, pH 9·6, ionic strength 0·02.

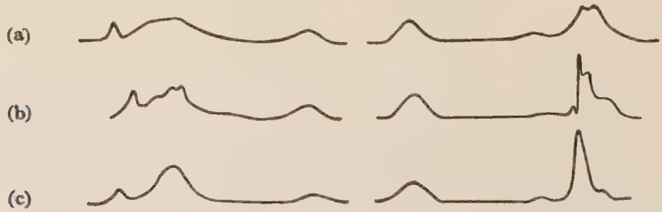


Fig. 5.—Electrophoresis in glycine buffer of ionic strength 0·1 and pH 11·0 of *S*-sulphokerateines prepared by urea-sodium bisulphite extraction at pH 5·0, followed by reaction with cuprammonium sulphite at pH 10·0. (*a*) First 15 per cent. of the protein extracted at pH 5·0 by 8M urea–sodium bisulphite; (*b*) the next 20 per cent. of the protein extracted at pH 5·0 by 8M urea–sodium bisulphite; (*c*) the next 20 per cent. of the protein extracted at pH 10·0 by cuprammonium sulphite.

eliminates some of the problems associated with the electrophoresis of –SH proteins. It is not then necessary to incorporate thioglycollic acid in the buffers and the descending patterns are free from some of the anomalous effects which occurred with the kerateines.

The use of sodium dodecyl sulphate or 8M urea with alkaline thioglycollate for extraction of the protein does not alter the electrophoretic patterns of the isolated protein and has the advantage that the whole extraction can be carried out between 10·5 and 11·0 at room temperature whereas in the method of Gillespie and Lennox (1953, 1955*a*) a temperature of 50°C is used and an initial pH of >12 for the final extraction. However, prolonged dialysis is necessary to remove sodium dodecyl sulphate.

Table 1

MOBILITIES OF COMPONENTS EXTRACTED FROM WOOL

Electrophoreses carried out in glycine–NaOH buffer, ionic strength 0·1, pH 11·0

Method of Extraction	Descending Mobility* $\times 10^5$ (cm^2 sec^{-1} volt^{-1})			
Alkaline thioglycollate–8M urea (pH 11·0)	*7·0*,	8·7,	10·2,	11·3,
Alkaline thioglycollate–0·5 per cent. sodium dodecyl suphate (pH 11·0)	*7·0*,	8·6,	10·3,	11·6,
Alkaline thioglycollate (pH 12·3)	*6·3*,	8·2,	9·5	
Cuprammonium sulphite–8M urea (pH 10·0)	*6·7*,	8·7,	9·8	
Cuprammonium sulphite (pH 10·0)	*7·4*,	8·9,	10·5	11·4
Sodium bisulphite–8M urea (pH 5·0)	*7·0*,	8·4,	10·6,	14·6

*Main component italicized.

The cuprammonium sulphite method of Swan (1957) gives similar results to the thioglycollate method and can be adapted for the fractional extraction of components. Although a complete quantitative analysis of the electrophoretic patterns was not carried out it was observed that if the electrophoretic patterns of the *S*-carboxymethylkerateines and *S*-sulphokerateines were compared for the same percentage of wool extracted, then the area under the main peak was approximately the same percentage of the total area in each case. The descending mobilities at pH 11 of the components (Table 1) are very similar for all methods of extraction, the differences between the methods being no greater than variability between preparations using the same method of extraction. The mobility of the isolated SCMK2 was $7\cdot2\times10^{-5}$ cm^2 sec^{-1} volt^{-1} (unpublished data), while that of SSK2 was $7\cdot0\times10^{-5}$ cm^2 sec^{-1} volt^{-1} indicating that the main component obtained by two different extraction methods is similar as regards electrical charge. The isolated SSK2 showed a very diffuse descending boundary at low ionic strength (Fig. 4(*e*)) and this is probably a similar phenomenon to that observed by Gillespie (1956) for SCMK2 and other keratin derivatives at low ionic strength. The main disadvantage in the preparation of the *S*-sulphokerateines is the difficulty of removing the excess copper from the extracted protein and often dialysis for several days in the presence of disodium ethylenediaminetetra-acetate was necessary for complete removal. There was no evidence of any decomposition during electrophoresis of the *S*-sulphokerateines at pH 11 although Swan (1957) reports a small amount of decomposition of the protein $-SSO_3^-$ group at this pH.

Examination of proteins extracted fractionally at pH 5·0 with urea bisulphite and subsequent stabilization of the –SH group by conversion to $-SSO_3^-$ shows that the proteins extracted exhibit similar electrophoretic behaviour to those extracted at higher pH values with other reducing agents. For small amounts of protein extracted the electrophoretic patterns are very similar to the first alkaline thioglycollate extracts obtained by Gillespie and Lennox (1955*a*). It appears that the order in which soluble proteins are extracted from wool is independent of the reagents used to reduce the disulphide bond or the pH of the reduction. Similar considerations would be expected to apply to the isolation of proteins from oxidized wool by the method of Alexander (Alexander and Hudson 1954). The electrophoresis experiments on *S*-sulphokerateines prepared from urea bisulphite extracts of wool are also of interest in that they show that the first extracts are more heterogeneous electrophoretically than the later extracts, whereas Friend and O'Donnell (1953) found that the osmotic molecular weights of urea bisulphite extracts were the same irrespective of whether 20 or 70 per cent. of the wool dissolved.

Interpretation of the Electrophoretic Patterns of Wool Proteins

In this and other work from this Laboratory concerning the electrophoresis of wool proteins there are many unexplained features of the electrophoresis diagrams. For example, descending and ascending patterns of the *S*-carboxymethyl proteins given in Figure 1 are not enantiographic. At low ionic strength the descending pattern of *S*-sulphokerateine 2 (Fig. 4(*e*)) is extremely spread out and does not correspond with the ascending pattern. Buffer ion effects certainly play some part in determining the type of patterns shown in Figure 1 where at pH 11 different patterns are obtained if carbonate or piperidine buffers are used instead of glycine. In the situation where thioglycollic acid is incorporated in the electrophoresis buffer to keep the protein in the reduced state (Gillespie and Lennox 1955*a*, 1955*b*), thioglycollate–protein interaction is a complicating factor and would be difficult to disentangle from protein-protein and other buffer ion–protein interactions. The thioglycollate–protein interaction is avoided, however, by stabilizing the proteins by substitution of the –SH group. With the more homogeneous kerateine derivatives prepared by Gillespie (1956), some peculiar descending electrophoretic patterns have been observed. The "twinning" of the main descending peak on the electrophoresis of the combined extracts and of the more homogeneous SCMK2 (reported in this work) in 0·1 ionic strength glycine buffer at pH 11 also needs some explanation. In view of the fact that physical measurements have shown that soluble wool proteins exist in solution in an aggregated state (O'Donnell and Woods 1956*a*, 1956*b*; Harrap and Woods 1958) the effect of reversible aggregation on the electrophoretic patterns is discussed below.

In order to fully interpret the electrophoretic patterns of a monomer in equilibrium with a series of polymers it is necessary to know, firstly, the relation between the electrophoretic mobility of a monomer and its polymers and, secondly, the time taken for equilibrium to be established compared to the time of electrophoresis since this determines the type of pattern.

There is no general answer to the first question. The relation between the electrophoretic mobility and net charge of solid spherical non-conducting spheres has been given by Henry (1931) and the effects of ionic strength of the medium and size and shape of the particle have been clearly set out by Abramson, Moyer, and Gorin (1942). From these relations it is possible to calculate the effect of linear aggregation on the electrophoretic mobility if it is assumed that the net charge and volume are increased n-fold when n monomer units aggregate linearly, and that for linear aggregation of spherical particles the polymer is assumed to be a cylinder. The results of such calculations for the range of sizes in which most proteins fall show that dimerization leads to little change in mobility and resolution would not be expected in a mixture of monomer and dimer. The calculated mobility increases slightly on further aggregation. No predictions can be made for the more likely case of non-linear aggregation since spheres and cylinders are the only models that can be treated by the above method. Recently, however, the electrophoresis of polyelectrolytes has been treated theoretically by Hermans and Fujita (1955) and Overbeek and Stigter (1956). At sufficiently high salt concentration polyelectrolyte molecules behave as free-drained on electrophoresis and the mobility should be independent of molecular weight and ionic strength. At lower ionic strengths polyelectrolytes becomes less free-drained; however, the effect of molecular weight on mobility is not very pronounced. Independence of mobility on molecular weight has been observed experimentally for poly-4-vinyl-N-n-butyl pyridinium bromide (Fitzgerald and Fuoss 1954) and polyphosphates (Strauss, Woodside, and Wineman 1957). In some proteins which dissociate, e.g. haemocyanin (Svedberg 1937) and ground-nut globulins (Johnson, Shooter, and Rideal 1950; Johnson and Naismith 1953), the rates of migration were shown to be independent of the degree of association. In studies on α-keratose (O'Donnell and Woods 1956*b*) prepared from wool it was found that resolution was obtained in the ultracentrifuge the results suggesting that much reversible aggregation occurred in solution, whereas on electrophoresis only a single peak was observed. These results are explained if these proteins behave on electrophoresis as free-drained or almost free-drained.

For cases where a polymer migrates faster than the monomer then normal resolution will take place if the equilibrium is established slowly compared to the time of electrophoresis. For times of equilibrium which are fast or comparable to the time of electrophoresis then a readjustment of equilibrium takes place during electrophoresis. A theoretical treatment of the effects of reversible polymerization during electrophoresis or ultracentrifugation has been given by Gilbert (1955) for the case where equilibrium is instantaneous. Where dimerization occurs a single peak is obtained. For a higher degree of polymerization a partly resolved boundary indicating two components will be formed. The situation is more complicated where a group of complexes each of different degree of polymerization is present, but the same principles are applicable and additional incompletely resolved boundaries would be expected.

Possibly the patterns obtained with kerateine derivatives at low ionic strength and high protein concentration could be explained by the presence of a monomer and its aggregates in equilibrium (Gillespie and Lennox 1955*b*; Gillespie 1956) if the assumption is made that the polymers migrate faster than the monomers. The latter assumption is doubtful. The complex boundaries formed by β_1-lactoglobulin on electrophoresis have been attributed to polymerization (Tombs 1957). In the case of this protein, phase rule solubility tests show it to be homogeneous whereas the purest wool protein prepared is heterogeneous by this test (Gillespie 1957). Hence it seems safer to conclude that the anomalous electrophoretic patterns arise from the heterogeneity of the materials although buffer ion interactions are not excluded as a contributing factor. Caution is needed in the interpretation of electrophoretic patterns of mixtures of components which interact since Colvin and Briggs (1952) have shown that the presence of reproducible shoulders or inflexion points in the schlieren diagrams is not always due to the presence of additional distinct components.

V. Acknowledgments

The author wishes to thank Dr. S. J. Leach for the cystine analyses on the extracted proteins and Miss B. F. Greening and Mr. L. West for assistance with the electrophoresis runs.

VI. References

Abramson, H. A., Moyer, L. S., and Gorin, U. H. (1942).—"Electrophoresis of Proteins." (Reinhold: New York.)

Alexander, P., and Hudson, R. F. (1954).—"Wool: Its Chemistry and Physics." (Chapman and Hall: London.)

Colvin, J. R., and Briggs, D. R. (1952).—*J. Phys. Chem.* **56**: 717.

Fitzgerald, E. B., and Fuoss, R. M. (1954).—*J. Polymer Sci.* **14**: 329.

Friend, A. J., and O'Donnell, I. J. (1953).—*Aust. J. Biol. Sci.* **6**: 630.

Gilbert, G. A. (1955).—*Disc. Faraday Soc.* **20**: 68.

Gillespie, J. M. (1956).—Proc. Int. Wool. Text. Res. Conf. Aust. 1955. Vol. B. p. B-35.

Gillespie, J. M. (1957).—*Aust. J. Biol. Sci.* **10**: 105.

Gillespie, J. M. (1958).—*Biochem. Biophys. Acta* **27**: 225.

Gillespie, J. M., and Lennox, F. G. (1953).—*Biochem. Biophys. Acta* **12**: 481.

Gillespie, J. M., and Lennox, F. G. (1955*a*).—*Aust. J. Biol. Sci.* **8**: 97.

Gillespie, J. M., and Lennox, F. G. (1955*b*).—*Aust. J. Biol. Sci.* **8**: 378.

Goddard, D. R., and Michaelis, L. (1935).—*J. Biol. Chem.* **112**: 361.

Harrap, B. S., and Woods, E. F. (1958).—*Aust. J. Chem.* **11**: 581.

Henry, D. C. (1931).—*Proc. Roy. Soc. Lond.* A **135**: 106.

Hermans, J. J., and Fujita, H. (1955).—*Proc. Acad. Sci. Amst.* B **58**: 182.

Johnson, P., and Naismith, W. E. F. (1953).—*Disc. Faraday Soc.* **13**: 98.

Johnson, P., Shooter, E. M., and Rideal, E. (1950).—*Biochem. Biophys. Acta* **5**: 376.

Lindley, H. (1955).—*J. Amer. Chem. Soc.* **77**: 4927.

Markus, G., and Karush, F. (1957).—*J. Amer. Chem. Soc.* **79**: 134.

O'Donnell, I. J., and Woods, E. F. (1956*a*).—*J. Polymer Sci.* **21**: 397.

O'Donnell, I. J., and Woods, E. F. (1956*b*).—Proc. Int. Wool. Text. Res. Conf. Aust. 1955. Vol. B. p. B-71.

Overbeek, J. H. G., and Stigter, D. (1956).—*Rec. Trav. Chim.* **75**: 543.

Strauss, M. P., Woodside, D., and Wineman, P. (1957).—*J. Phys. Chem.* **61**: 1353.

Svedberg, T. (1937).—*Chem. Rev.* **20**: 81.

Svensson, H. (1946).—*Ark. Kemi. Min. Geol.* A **22**: 10.

Swan, J. M. (1957).—*Nature* **180**: 643.

Tombs, M. P. (1957).—*Biochem. J.* **67**: 517.

Woods, E. F. (1952).—*Aust. J. Sci. Res.* A **5**: 555.

Woods, E. F. (1958).—*J. Phys. Chem.* **62**: 308.

THE THEORY OF SOME GENETICAL EFFECTS OF POPULATION SUBDIVISION

By P. A. P. Moran*

[*Manuscript received September* 26, 1958]

Summary

The genetical effects of the subdivision of a population into partially isolated subgroups are considered in two particular cases. In the first a probability model is studied in which the subpopulations are of finite size with migration between them. In the absence of selection the asymptotic rate of progress to homozygosity is shown to be very little affected by the subdivision. In the second case a deterministic model is studied in which there are two subpopulations in which selective forces are equal and opposite. A stable dimorphism is then shown to exist if there is any small amount of intermigration.

I. Introduction

The evolutionary implications of the subdivision of a population into groups which are partly isolated from one another has long been a subject of controversy. Wright and Fisher have shown that in a completely random mating population without selection or mutation the probability of the population remaining heterozygous with respect to two alleles at a single locus is proportional to λ^t, where t is measured in generations, and $\lambda = 1-N(8N_1\,N_2)^{-1}$. Here N_1 and N_2 are the numbers of males and females, $N = N_1+N_2$, the generations are non-overlapping, and the distribution of the number of offspring per parent is approximately Poissonian. The theory of this phenomenon is simpler if we consider a population of N monoecious individuals. If these are diploid the population is completely defined by the nature of the $2N$ gametes from which they were formed. The probability of the population remaining heterozygous is then asymptotically proportional to λ^t where $\lambda = 1-(2N)^{-1}$.

It is known that inbreeding resulting from assortative mating or consanguinity can alter this rate considerably. If now we consider a population spread out on a wide geographical area, the conditions of strict random mating can no longer hold and this will be still more evident in a population divided into subgroups between which only a limited amount of migration takes place. It is therefore of some interest to study mathematical models of such subdivided populations.

We could do this for populations of diploid individuals with sexual differentiation but the algebra is then so complicated that we confine ourselves to monoecious populations and thus it is only necessary to consider populations of haploid individuals. This will give a quite adequate idea of the effects of migrations between subpopulations in more complicated models.

---

* Department of Statistics, Australian National University, Canberra.

II. Random Approach to Homozygosity in a Subdivided Population

Suppose then that we have $H+1$ subpopulations each of N haploid individuals. The total population thus consists of $(H+1)N$ individuals and if there were no subdivisions the probability of heterozygosity would decrease asymptotically as $\{1-[(H+1)N]^{-1}\}^t$. We suppose, however, that in each subpopulation the next generation contains K migrants chosen at random from the offspring of each of the other H populations, together with $N-HK$ offspring from the original subpopulation itself. We write $L = HK$ for the total number of migrants into each subpopulation. Then $L < N$. Each of these migrants is a haploid individual.

In this model we have assumed that migrants come equally from all the other subpopulations. A more realistic model would be obtained if we assume that the subpopulations are distributed geographically over a two-dimensional area, for example if they were concentrated at the vertices of a rectangular lattice. Migration would then be permitted only from the nearest neighbouring subpopulations. Difficulties about the special position of subpopulations on the "edge" of the lattice area could be obviated by wrapping the lattice around a torus. However, even in this case the algebra becomes unwieldy. It is therefore necessary to confine ourselves to the case where each subpopulation receives the same number of migrants from each of the other subpopulations.

We consider a single locus with two possible alleles, a and A. We suppose that in generation t the number of a individuals in the ith subpopulation ($i=1, \ldots, H+1$) is k_{ti} so that the number of A individuals is $N-k_{ti}$. We can then write

$$k_{ti} = k_{tii} + \sum_{j}^{i*} k_{tij}, \qquad \text{....................(1)}$$

where k_{tii} is the number of a individuals which are descended from $k_{(t-1)i}$ and k_{tij} the number of migrant a individuals in population i descended from $k_{(t-1)j}$. The symbol $\sum_{j}^{i*}$ is used to signify summation over all values of $j \neq i$.

The generations are taken as non-overlapping and the offspring from the ith population are taken to be a or A independently with probabilities $k_{ti}N^{-1}$ and $(N-k_{ti})N^{-1}$. To calculate the asymptotic rate of progress to homozygosity we set up recurrence relations between the moments of the variates k at times t and $t+1$. For simplicity of notation we write $t = 0$. We then have

$$E(k_{1ii}) = (N-L)N^{-1}k_{0i},$$

$$E(k_{1ij}) = KN^{-1}k_{0j}, \qquad (i\neq j)$$

$$\operatorname{var}(k_{1ii}) = (N-L)N^{-2}k_{0i}(N-k_{0i}),$$

$$\operatorname{var}(k_{1ij}) = (N-L)N^{-2}k_{0j}(N-k_{0j}).$$

These are expectations at time $t = 1$ conditional on fixed values of the k's at time $t = 0$. It follows that the k_{1ii} and the k_{1ij} are independent and therefore

$$\operatorname{var}(k_{1i}) = (N-L)N^{-2}k_{0i}(N-k_{0i}) + \sum_{j}^{i*} KN^{-2}k_{0j}(N-k_{0j}),$$

$$\operatorname{var}(\sum_{i} k_{1i}) = N^{-1} \sum k_{0i}(N-k_{0i}).$$

We now write $k_t = \sum_i k_{ti}$. Then

$$\begin{aligned} E(k_1) &= \sum E\, k_{1i} = \sum E\left\{k_{1ii} + \sum_{j}^{i*} k_{1ij}\right\} \\ &= N^{-1} \sum_{i}\left\{(N-L)k_{0i} + \sum_{j}^{*i} K\, k_{0j}\right\} \\ &= \sum k_{0i} \\ &= k_0. \end{aligned}$$

Put

$$\begin{aligned} A_t &= \sum_{i} E(k_{ti}^2), \\ B_t &= \sum_{i} \sum_{j}^{i*} E(k_{ti}\, k_{tj}), \\ C_t &= \sum_{i} E(k_{ti}). \end{aligned}$$

Then from above, $C_t = C_0$ and is constant. We also have

$$\begin{aligned} E(k_{1i}^2) &= (E\, k_{1i})^2 + \operatorname{var}(k_{1i}) \\ &= N^{-2}\left\{(N-L)k_{0i} + K \sum_{j}^{*i} k_{0j}\right\}^2 \\ &\quad + N^{-2}\left\{(N-L)k_{0i}(N-k_{0i}) + \sum_{j}^{*i} K\, k_{0j}(N-k_{0j})\right\}. \end{aligned}$$

Multiplying out and summing over all i we find

$$A_1 = N^{-2}\{(N-L)^2 + KL - N\}A_0 + N^{-2}\{2NK - KL - K^2\}B_0 + C_0. \quad \ldots . (2)$$

Next we have, for $i \neq j$,

$$E(k_{1i}\, k_{1j}) = N^{-2}\left\{(N-L)k_{0i} + K \sum_{l}^{*i} k_{0l}\right\}\left\{(N-L)k_{0j} + K \sum_{m}^{*j} k_{0m}\right\}.$$

Multiplying out and summing we get

$$\begin{aligned} B_1 &= \sum_{i} \sum_{j}^{*i} E(k_{1i}\, k_{1j}) \\ &= N^{-2}\{(N-L)^2 B_0 + 2(N-L)KHA_0 + 2(N-L)K(H-1)B_0 + K^2 B_0 \\ &\qquad + 2K^2(H-1)B_0 + K^2 H(H-1)A_0 + K^2(H-1)(H-2)B_0\} \\ &= N^{-2}\{N^2 - 2NK + LK + K^2\}B_0 + N^{-2}\{2NL - L^2 - KL\}A_0. \end{aligned}$$

The process may be regarded as a Markov chain in which the state of the system is determined by the $(H+1)$ variables $k_{ti}(i = 1, \ldots, H+1) = 0, \ldots . N$. There are exactly two absorbing states for which all the k_{ti} are zero, or all equal to N, and from any other state all states are accessible. The matrix of transition probabilities is far too complicated to be written down explicitly but we may determine the moduli of its three largest roots which we write as λ_0, λ_1, and λ_2. Clearly $\lambda_0 = \lambda_1 = 1$ and there are no other roots of unit modulus. Starting from any non-absorbing state the probability of any other non-absorbing state at generation t will be asymptotically equal to a constant multiple of $|\lambda_2|^t$ and the probabilities of the two absorbing states will be approximately of the form $\alpha - \beta|\lambda_2|^t$. Thus the expression

$$T = E\,\Sigma(k_{tt} - \tfrac{1}{2}N)^2, \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

will be asymptotically increasing and of the form $\gamma - \delta|\lambda_2|^t$ where $\delta > 0$. Thus since T can be expressed in terms of A_t and C_t we can find $|\lambda_2|$ by picking out the largest root of the matrix of coefficients in the set of difference equations expressing A_{t+1}, B_{t+1}, and C_{t+1} in terms of A_t, B_t, and C_t.

TABLE 1

VALUES FOR ν

K	H			
	1	2	4	8
1	0·8769	0·8953	0·9246	0·9527
2	0·9378	0·9460	0·9612	0·9752
4	0·9688	0·9726	0·9803	0·9878
8	0·9844	0·9862	0·9901	0·9939
16	0·9922	0·9930	0·9950	0·9970
100	0·9988	0·9989	0·9992	0·9994

Writing $\lambda = 1 - \mu N^{-1}$, we have to find the smallest root in μ of the equation

$$\begin{vmatrix} \mu N - (2NL - L^2 - KL + N) & 2NK - KL - K^2 \\ 2NL - KL - L^2 & \mu N - (2NK - KL - K^2) \end{vmatrix} = 0$$

and this becomes, on division by N,

$$-\mu^2 N - \mu\{(K+L)(2N-K-L)+N\} + K(2N-K-L) = 0. \qquad \ldots(4)$$

The total population size is $(H+1)N$ haploid individuals and if there were no subdivision of the population the largest non unit root would be $1 - \{(H+1)N\}^{-1}$ which would govern the asymptotic rate of approach to homozygosity. To estimate the effect of subdivision on this root we write

$$\lambda = 1 - \mu N^{-1} = 1 - \nu\{(H+1)N\}^{-1},$$

and calculate ν as a function of H and K. As K gets large we expect ν to approach unity. Table 1 gives ν for selected values of H and K, and shows that the effects of subdivision on the asymptotic rate of progress to homozygosity is small and rapidly becomes negligible when K, the number of migrants exchanged between each subpopulation, increases beyond one or two. In fact, it is easily verified that for

$$\lambda = 1-\nu\{(H+1)N\}^{-1},$$

ν rapidly tends to unity as H or K increases, for the asymptotic form of equation (4) is

$$\mu^2-\mu\{2(H+1)K+1\}+2K = 0.$$

and the relevant root of this is nearly $(H+1)^{-1}$ when H or K is large.

These results show that geographical subdivision will have only a very small affect on the asymptotic rate of drift to homozygosity in small populations between which there is some small amount of intermigration. This conclusion, however, is dependent on there being no selective effects. The problem of the effect of selection on the rate of progress to homozygosity is a very difficult one and has been discussed for a single population by Wright and Kerr (1954) and by Kimura (1955). These papers estimate the result of a small selective effect, without dominance, on the principal root and show that the asymptotic rate of progress to homozygosity is increased.

III. Stable Polymorphism in a Subdivided Population

The situation is entirely changed if we suppose that the population is divided into two parts in which selection operates in different directions. To discuss the theory of a stochastic model of this kind would be a very difficult undertaking. We shall confine ourselves to considering a deterministic model and show that there exists a stable state in which both alleles exist, i.e. a stable polymorphism similar to the well-known case in which the heterozygote has a selective advantage.

Consider two populations within each of which mating is at random so that we can represent the frequencies of the zygotes aa, Aa, and AA by p_1^2, $2p_1q_1$, q_1^2, and p_2^2, $2p_2q_2$, q_2^2 respectively. Next suppose that in the first population the relative reproductive powers of the zygotes are in the ratios $(1+m)$: 1 : $(1-m)$. Similarly in the second population we suppose they are in the ratios $(1-m)$: 1 : $(1+m)$, i.e. we assume that selection operates equally but in opposite directions. Then the frequency of gene a in the offspring from the first population will be

$$\frac{(1+m)p_1^2+2p_1q_1}{(1+m)p_1^2+2p_1q_1+(1-m)q_1^2},$$

and if m is small this will be equal to $p_1+mp_1q_1$. Similarly the a gene frequency in the second population will be $p_2-mp_2q_2$. We now suppose that the next generation in each subpopulation consists of a fraction $(1-k)$ of descendants of this population and a fraction k which are descendants of the other population. In general k will be small but for the following results to hold it is necessary only that $k<\frac{1}{2}$. If the values of the frequencies of gene a in the new generation are p_1' and p_2' we then have, supposing m small,

$$p_1' = p_1+k(p_2-p_1)+(1-k)mp_1q_1-mkp_2q_2,$$

$$p_2' = p_2+k(p_1-p_2)-(1-k)mp_2q_2+mkp_1q_1.$$

We first consider what values P_1, P_2 of p_1 and p_2 will result in a stationary state, i.e. one for which $p_1' = p_1$ and $p_2' = p_2$. Writing $l = k^{-1}$ we obtain from the above equations

$$P_2-P_1+(l-1)mP_1Q_1-mP_2Q_2 = 0,$$
$$P_1-P_2-(l-1)mP_2Q_2+mP_1Q_1 = 0,$$

where $Q_1 = 1-P_1$, $Q_2 = 1-P_2$.

Adding we see that $P_1Q_1 = P_2Q_2$. Thus we must have $P_1 = P_2$ or $P_1 = Q_2$. If $P_1 = P_2$ we must have $(l-2)P_1Q_1 = 0$ and so $P_1Q_1 = P_2Q_2 = 0$ so that either $P_1 = P_2 = 0$ or $P_1 = P_2 = 1$. Both of these are possible solutions. If $P_1 = Q_2$ we substitute for P_2 and Q_2 in the first equation and find

$$P_1^2m(2-l)-P_1\{m(2-l)+2\}+1 = 0,$$

and since $l > 2$ we see that the root

$$\tfrac{1}{2}+\{m(2-l)\}^{-1}-\sqrt{\{\tfrac{1}{4}+m^{-2}(2-l)^{-2}\}}$$

is less than zero and so is not relevant. The other root

$$\tfrac{1}{2}+\{m(2-l)\}^{-1}+\sqrt{\{\tfrac{1}{4}+m^{-2}(2-l)^{-2}\}}$$

lies between $\frac{1}{2}$ and 1 and results in a stationary solution.

Consider the stability of solution $P_1 = P_2 = 0$. For p_1 and p_2 small we have the approximate equations

$$p_1' = p_1(1-k)(1+m)+p_2k(1-m),$$

$$p_2' = p_1k(1+m)+p_2(1-k)(1-m).$$

These are a pair of linear difference equations and the population will be stable in the neighbourhood of $p_1 = p_2 = 0$ if, and only if, the roots of the determinantal equation

$$\begin{vmatrix} \lambda-(1-k)(1+m) & -k(1-m) \\ -k(1+m) & \lambda-(1-k)(1-m) \end{vmatrix} = 0,$$

are less than unity in absolute value. The roots are

$$\lambda = (1-k)\pm\sqrt{\{k^2+m^2(1-2k)\}}.$$

Since we have assumed $k < \frac{1}{2}$ the term inside the radical is certainly greater than k^2 and thus one root is greater than $1-k+k = 1$. The solution $P_1 = P_2 = 0$ is therefore unstable. Similarly the solution $P_1 = P_2 = 1$ is unstable by symmetry.

For the third solution we have $\frac{1}{2} < P_1 = Q_2 < 1$. Write $P_1 = Q_2 = P = 1-Q$ say, and

$$p_1 = P + \delta_1, \qquad q_1 = Q - \delta_1,$$
$$p_2 = Q + \delta_2, \qquad q_2 = P - \delta_2,$$

where δ_1, δ_2 are small. Expanding the recurrence relation about the point (P, Q) we get

$$\delta_1' = \delta_1(1-k)(1+m(Q-P))+\delta_2 k(1+m(Q-P)),$$
$$\delta_2' = \delta_1 k(1+m(Q-P))+\delta_2(1-k)(1+m(Q-P)).$$

The state (P, Q) will be stable if, and only if, the roots of the determinantal equation

$$\begin{vmatrix} \lambda-(1-k)(1+m(Q-P)) & k(1+m(Q-P)) \\ k(1+m(Q-P)) & \lambda-(1-k)(1+m(Q-P)) \end{vmatrix}$$
$$= \lambda^2-2(1-k)(1+m(Q-P))\lambda+(1-2k)(1+m(Q-P))^2 = 0,$$

are less than unity in absolute value.

These roots are

$$\lambda = 1+m(Q-P), \quad (1-2k)(1+m(Q-P)),$$

and since $P > Q$ and $k < \frac{1}{2}$ both these roots are less than unity so that a stable polymorphism exists. This model could be generalized without much difficulty to a situation in which selection in the two subpopulations is unequal so long as they are of opposite signs, and similarly the migration intensities could be made unequal.

Levene (1953) has considered a similar model with several subpopulations but supposes the individuals to leave their niches immediately before breeding thus forming one large panmictic population whose offspring are later distributed amongst the different niches.

Thus a deterministic model results in the existence of a stable polymorphism. If we take into account the fact that the populations are finite, the ultimate fate of the population is quite different since there are two absorbing states and one of these must ultimately be attained. The stable state with $0 < P_1 < 1$ now has only a quasi-stable character in that there will be a strong tendency for the population to return to it when disturbed. Thus in the absence of mutation the rate of approach to homozygosity will be very much slower than that which would occur if there were no selection. Unfortunately the problem of calculating this rate appears to be very difficult.

The evolutionary significance of these results is that "drift" to homozygosity in small populations may be very much slowed down if the population contains two niches in which selection operates in opposite directions. This will be true even for two populations between which there is only a small amount of migration. However, in practice, we are unlikely to have niches which differ in their selective effects for only a single character and it may well happen that the accumulation of genetic differences between the two subpopulations finally results in barriers which reduce and ultimately eliminate cross-breeding.

IV. References

Kimura, M. (1955).—Stochastic processes and gene frequencies. *Cold Spring Harb. Symp. Quant. Biol.* **20**: 33–53.

Levene, H. (1953).—Genetic equilibrium when more than one ecological niche is available. *Amer. Nat.* **27**: 331–3.

Wright, S., and Kerr, W. E. (1954).—Experimental studies in the distribution of gene frequencies in very small populations of *Drosophila melanogaster*. II. *Evolution* **8**: 225–40.

A STUDY OF HIGH TEMPERATURE LESIONS IN *ARABIDOPSIS THALIANA*

By J. Langridge* and B. Griffing*

[*Manuscript received November* 10, 1958]

Summary

The composite hypothesis under test is that (i) at certain temperature extremes plant growth is depressed by the inactivation of one or a few especially sensitive reactions, and (ii) such growth depression may be prevented by providing the plant with the normal products of the inhibited reactions. Appropriate experimental designs and statistical criteria to test this hypothesis are formulated.

Tests were conducted using homozygous ecotypes (races) of *Arabidopsis thaliana* grown aseptically in growth chambers. It was found that the genotypic component of the measurable growth variation between races is positively correlated with temperature. Therefore, to detect races particularly sensitive to high temperatures, 43 races were grown at 25, 30, and 31·5°C. Eight races showed a disproportionately great decrease in growth at 31·5°C, five of these possessing pronounced morphological symptoms of high temperature damage. Three of these five races gave significantly increased growth at 31·5°C with vitamins, yeast extract, and nucleic acids respectively. The specific stimulating substance for the first two races was found to be biotin, which completely prevented the formation of the temperature lesion; in the third race, a partial alleviation of the temperature lesion resulted from the addition of cytidine.

Preliminary genetic tests of a non-responding temperature-lesion race suggested that the extreme temperature sensitivity is polygenically determined, although it is probable that the three races requiring specific metabolites differ from others primarily at single loci.

It is concluded that the results support the hypothesis, and that the phenomenon may be one of importance in determining the climatic tolerance of cultivated plants.

I. Introduction

The possibility of the "chemical cure of climatic lesions" has been proposed by Bonner (1957). This proposal is particularly interesting because of its considerable potential economic importance. Inherent in the concept of a chemical cure of climatic lesions are the following two basic hypotheses: (i) That at certain climatic extremes, there may be inactivation of one or a few reactions contributing to growth. These reactions are detectable by experiment because they are especially sensitive to the environmental factor that is being intensified. (ii) That the products of these few reactions are essential metabolites which may be introduced into the organism's cellular economy from external sources.

The hypothesis, as it applies to temperature effects, is presented in quantitative form, together with the appropriate interaction tests, in Section III. Using these formulations, the data of Bonner (1943) and Galston and Hand (1949), which

---

*Division of Plant Industry, C.S.I.R.O., Canberra.

represent the main experimental basis for the hypothesis with respect to temperature, have been re-analysed.

Bonner (1943) reported a set of experiments in which thiamine was applied to *Cosmos* plants grown in different temperature regimes. The responses obtained were taken to indicate a case of a chemically reparable low temperature lesion (Bonner 1957). As a basis for this inference, Bonner, using dry weights, compared, on the arithmetic scale, the control response with that of the supplement at each temperature. No interaction tests were performed.

In our analyses of these data we found that only one experiment (C67), of four factorial-type experiments (C60, C66, C67, and C81) yielded a significant interaction effect when the analyses were based on an arithmetic or logarithmic scale of measurement. However, on both scales of measurement, a pooled test involving all four experiments gave a significant admissible interaction response. Hence we conclude that the data, collectively, support the hypothesis.

Galston and Hand (1949) presented data which were taken by the authors to provide evidence of a high-temperature-induced reduction of the ability of the pea plant to synthesize adenine. The pertinent results were obtained from experiments on the addition of adenine alone, and in the presence of indoleacetic acid, to subapical sections of etiolated pea epicotyls at 25 and 35°C. No statistical tests of the hypothesis were made.

Our analyses of their data were made with each of two methods of measuring growth. We considered (1) the increments of growth of the sections measured on the arithmetic scale, and (2) the logarithmic transformation of the total final length of the section (i.e. total length = initial length + increment). In both cases the interaction effects were small and non-significant (arithmetic, $t_{44} = 0\cdot66$; logarithmic, $t_{44} = 0\cdot40$). Thus, there is apparently no conclusive evidence to support the hypothesis that the elevated temperature induces a deficiency in adenine.

Galston (1957) has reported the response to adenine of intact green pea plants. All plants were kept at 23°C during the day but different groups were subjected to different night temperatures ranging from 2 to 30°C. The resultant data are reported in condensed graphical form and no measurements of variability are given. Therefore, it has not been possible to analyse the data in a factorial manner. The responses listed for stem height and stem fresh weight are less pronounced than those for leaf fresh weight, where adenine appears to be exerting its greatest effect. However, even in this case, the stimulation by adenine is much the same over the entire temperature range, indicating that temperature is not inducing an adenine deficiency.

The above re-analyses indicated that the evidence so far presented for the occurrence of temperature lesions is not entirely conclusive. Because of this lack of critical data, it was considered worth while to conduct experiments especially designed to test the hypothesis.

II. Material and Methods

As a source of natural variability, 43 races of *Arabidopsis thaliana* (L.) Heynh. were used. These races, from the collection of Professor F. Laibach, Frankfurt-am-Main, were originally collected in the geographical area extending from Western

Europe (Hilversum) to Asia (Tsu Islands of Japan) and from Northern Europe (Stockholm) to North Africa (Martuba) (Table 1). All plants of each race used in the experiments were grown from seeds of a single plant. Because they are obligately self-fertilized, individual plants may be regarded as homozygous.

Plants were grown aseptically in test tubes using the cultural methods described previously (Langridge 1957). The plants of each experiment were grown in continuous fluorescent light of approximately 1200 foot-candles intensity in an atmosphere of 70 per cent. relative humidity. The growth chambers were especially manufactured for the study of temperature effects on plant growth by the Engineering Section, C.S.I.R.O., Melbourne.

TABLE 1

LIST OF THE RACES OF ARABIDOPSIS THALIANA USED IN THE EXPERIMENTS

Race Abbreviation	Origin of Race		Race Abbreviation	Origin of Race	
	Place	Country		Place	Country
BD	Berlin-Dahlem	Germany	KOP	Kopenhagen	Denmark
BE	Bensheim	Germany	KS	Kassel-Döncke	Germany
BLA	Blanes	Spain	LA	Landsberg-am-Warthe	Germany
BO	Bonn	Germany			
BR	Brünn	Czechoslovakia	LI	Limburg	Germany
BS	Basel	Switzerland	LS	Langen-Sprendlingen	Germany
CHI	Schisdra	Russia	LU	Lund	Sweden
CT	Catania	Sicily	MT	Martuba	North Africa
DA	Darmstadt	Germany	PF	Pfrondorf	Germany
DI	Dijon	France	PI	Pitztal	Austria
DR	Dresden	Germany	RLD	Rschew	Russia
EI	Eifel	Germany	ROU	Rouen	France
EN	Enkheim	Germany	SCH	Schwanheim	Germany
ER	Erlangen	Germany	ST	Stockholm	Sweden
EST	Estland	Estonia	TA	Tabor	Czechoslovakia
FR	Frankfurt-am-Main	Germany	TS	Tsu Islands	Japan
GIE	Giessen	Germany	TU	Turin	Italy
GR	Graz	Austria	VI	Bad Vilbel	Germany
HI	Hilversum	Holland	WI	Wisselsheim	Germany
HM	Hannovrisch-Munden	Germany	WIL	Wilna	Lithuania
IN	Innsbruck	Austria	WK	Wachküppel	Germany
KOL	Köln	Germany	WS	Wassilewskija	Russia

Fresh weight was taken as a measure of growth because such weights may be rapidly and accurately obtained. The plants were pulled from the agar without loss of root tissue, moisture was removed from the roots by blotting, and the plant was immediately weighed. Because the plants are grown on an agar substrate which is 99 per cent. water and in an atmosphere of high and constant relative humidity, it is unlikely that growth responses will be obscured by variations in moisture content.

In the experiments reported, an agar temperature of 25°C was taken as the approximate optimum for the growth of the species.

In all experiments requiring analysis, both arithmetic and logarithmic fresh weight means are given; the analyses, however, were performed only on the logarithmic data. The logarithmic transformation was used since the data were derived from the exponential growth phase.

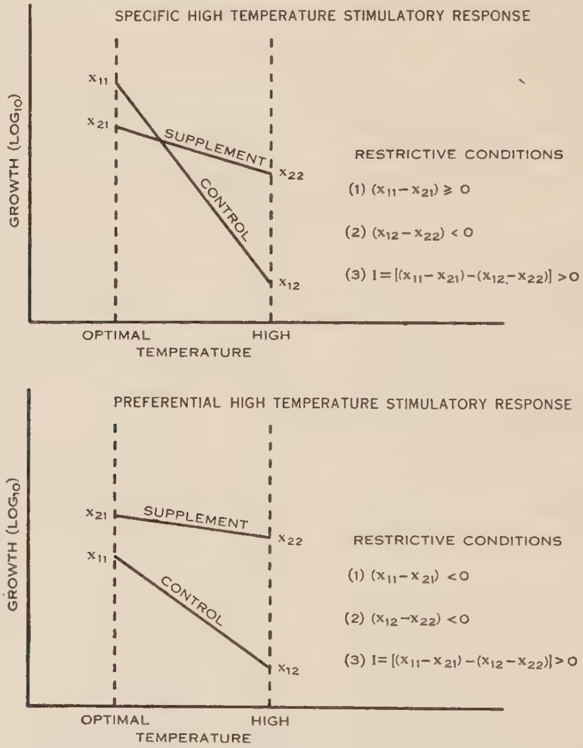

SPECIFIC HIGH TEMPERATURE STIMULATORY RESPONSE
GROWTH ($\log_{10}$)
x_{11}
x_{21}
SUPPLEMENT
CONTROL
x_{22}
x_{12}
RESTRICTIVE CONDITIONS
(1) $(x_{11}-x_{21}) \geq 0$
(2) $(x_{12}-x_{22}) < 0$
(3) $I=[(x_{11}-x_{21})-(x_{12}-x_{22})]>0$
OPTIMAL
HIGH
TEMPERATURE
PREFERENTIAL HIGH TEMPERATURE STIMULATORY RESPONSE
GROWTH ($\log_{10}$)
x_{21}
SUPPLEMENT
x_{22}
x_{11}
CONTROL
x_{12}
RESTRICTIVE CONDITIONS
(1) $(x_{11}-x_{21}) < 0$
(2) $(x_{12}-x_{22}) < 0$
(3) $I=[(x_{11}-x_{21})-(x_{12}-x_{22})]>0$
OPTIMAL
HIGH
TEMPERATURE

Fig. 1.—Graphical representations of high temperature stimulatory growth responses relevant to the Bonner hypothesis.

III. The Hypothesis with respect to Experimental Procedure

The hypothesis is, simply, that an extreme temperature (high or low) restricts growth by inducing a deficiency in an essential metabolite. Thus, in temperature-sensitive plants, a more pronounced deficiency in an essential metabolite exists at the extreme than at the optimal temperature. Experimentally, therefore, application of the appropriate supplement should induce a relatively greater increase in growth at the extreme temperature.

In order to detect and test for such an effect, the temperature-sensitive races must be grown, with and without the supplement, at both optimal and extreme temperatures. Such a factorial experiment leads to useful definitions and an exact test of the hypothesis.

These notions are more precisely formulated by considering high and low temperature lesions separately. We shall consider the situation with *high* temperature lesions in detail.

The elements in the following 2 × 2 table represent the means resulting from growing a given race, with and without a specific supplement, at both an optimal and a high temperature:

	Temperature	
	Optimal	High
Control	x_{11}	x_{12}
Supplement	x_{21}	x_{22}

Of the various possible responses, the only relevant ones are those in which $(x_{22}-x_{12})>0$, i.e. in which the specific supplement causes a growth stimulation at the high temperature. Experimentally, then, this criterion provides the first screening test with regard to the hypothesis.

An exact test and definition of the hypothesis is provided by the interaction contrast associated with the 2 × 2 table. This contrast may be defined as

$$I = [(x_{11}-x_{21})-(x_{12}-x_{22})].$$

The two types of stimulatory responses which fulfil the requirements of the hypothesis can be specified exactly by restrictions placed on I and its components, as given in Figure 1. Thus a "specific high temperature stimulatory response" is one in which the supplement causes a growth stimulation at the high temperature and causes no stimulation, or may even depress growth, at the optimal temperature. The other type of response, designated as a "preferential high temperature stimulatory response", is one in which the supplement causes an increase in growth at both temperatures, but the response at the high temperature is greater than at the optimum. The test of the hypothesis resides in determining whether or not the interaction contrast is significantly different from zero.

Definitions and a test for *low* temperature stimulatory responses may be formulated in a similar manner.

The above considerations suggest a logical procedure for an experimental study of the *A. thaliana* races. In the following steps it is assumed that the decision has been made to concentrate on high temperature, rather than low temperature, lesions:

(i) The entire collection of races is subjected to optimal and high temperatures to identify those races which are sensitive to high temperatures.

(ii) The selected high temperature sensitive races are grown with an extensive range of organic supplements at the high temperature. This allows an identification of those races which respond to supplements and also determines the specific supplement causing a growth stimulation for a given race.

(iii) A factorial experiment is conducted in which the responsive high temperature sensitive races are grown with and without the supplement at the optimal and high temperatures. The races may then be classified according to the type of response they manifest and the appropriate interaction test may be performed.

The actual experimental procedures followed in the experiments reported in this study are an elaboration of these basic steps.

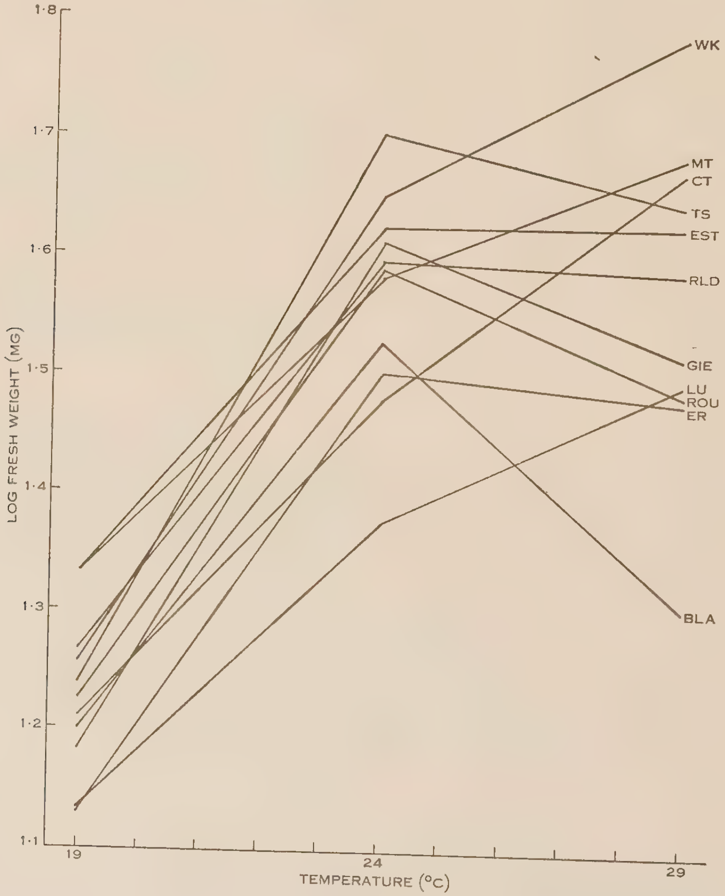


Fig. 2.—Growth of 11 races at three temperatures plotted as log fresh weights.

IV. Results

(a) *Experiment* 1

This preliminary experiment was designed to determine whether differences in growth between races would become apparent at high or low temperatures, or at both. Eleven races, chosen at random, were grown for 21 days at agar temperatures of 19, 24, and 29°C. Twenty seeds were planted for each race-temperature combination, but because of unequal germination the numbers of final observations for different race–temperature combinations varied. The plant numbers ranged from 9 to 20, with an average number of 16.

The means of the log fresh weight values for the 11 races at each temperature are plotted in Figure 2. It is immediately apparent that race differences in growth are most extreme at the high (29°C) temperature. This fact is more rigorously examined by variance component estimates in which the variance due to genotypic differences between races is isolated from the environmental variance. Therefore, an analysis of variance of the type "single classification with unequal numbers" has been calculated for the 11 races at each temperature. With the assumption that the races used are a random sample of a large population of possible races, such an analysis of variance provides (1) a test for differences among races, and (2) an estimation of genotypic and environmental components of variance.

Table 2

GENOTYPIC AND ENVIRONMENTAL VARIANCE COMPONENTS FOR 11 RACES AT THREE TEMPERATURES

Variance Component	Agar Temperature (°C)		
	19	24	29
Genotypic	0·0040	0·0082	0·0167
Environmental (error)	0·0096	0·0170	0·0201

Table 3

GENOTYPIC AND ENVIRONMENTAL VARIANCE COMPONENTS FOR 43 RACES AT THREE TEMPERATURES

Variance Component	Agar Temperature (°C)		
	25	30	31·5
Genotypic	0·0128	0·0115	0·0592
Environmental (error)	0·0407	0·0560	0·0711

The estimates of genotypic and environmental variance components are given in Table 2. Tests resulting from an analysis of variance yielded highly significant F values ($P<0·01$) for each of the three temperatures. Therefore, we may conclude that there are real genotypic differences between races which are manifest at each temperature. Since the genotypic variance is positively correlated with temperature, we may further conclude that genotypic differences in growth among races are most evident at the higher temperatures. Our attention in subsequent experiments has therefore been focused on growth responses at high temperatures.

(*b*) *Experiment* 2

In order to detect high temperature lesions, the entire collection of races at our disposal was grown for 14 days at agar temperatures of 25 and 30°C. Fifteen seeds of each of the 43 races were planted for each temperature treatment, but because of unequal germination the number of plants per treatment was reduced to 10. The mean log fresh weights for the races are set out in the scatter diagram of Figure 3.

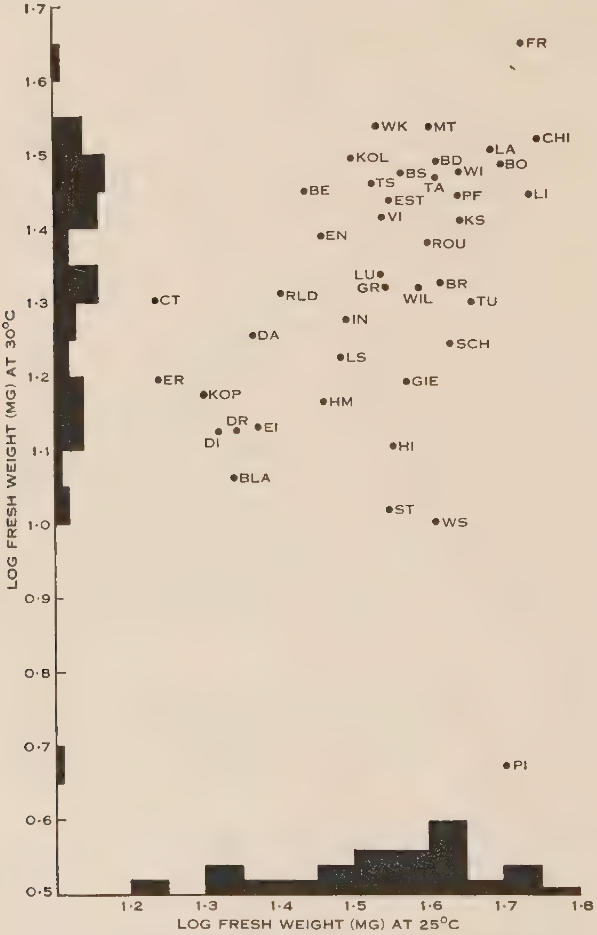


Fig. 3.—Scatter diagram of the log fresh weights of 43 races grown at 25 and 30°C.

The temperature of 30°C, however, seemed in most cases to be below the threshold necessary to produce pronounced deficiency symptoms. Therefore, the races were regrown at a slightly higher agar temperature, viz. 31·5°C. The scatter diagram of Figure 4 shows the mean log fresh weights at 31·5°C plotted against those at 30°C. The analysis of variance, given in Table 3, resulted in a highly significant ($P<0\cdot01$) mean square for race differences at each temperature.

The fact that the temperature of 30°C was not sufficiently high for the manifestation of most temperature lesions is evident when the estimated genotypic variance components are compared for 25 and 30°C. The appearance of high temperature lesions at 31·5°C can be detected quantitatively by the five-fold increase in the estimated genotypic variance component for this temperature.

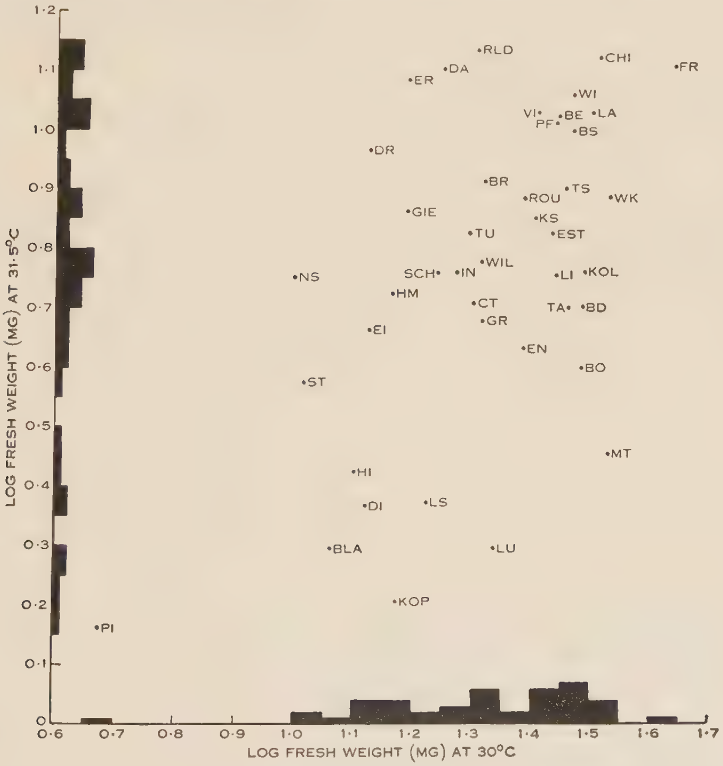


Fig. 4.—Scatter diagram of the log fresh weights of 43 races grown at 30 and 31·5°C.

These quantitative differences between races, as well as the joint distribution of races at different temperatures, are more conveniently depicted in the scatter diagrams (Figs. 3 and 4). These figures show that:

(i) With an increase of temperature to 30°C, the growth of the race PI drops from a very high to an exceptionally low value. This race is the first temperature-lesion race to appear.

(ii) At 31·5°C, at least eight races have appeared which form a high temperature lesion group.

The morphological symptoms of the high-temperature-induced growth depression were very pronounced and uniform in five of the eight races of the lesion group. The races LU, KOP, and MT showed no pronounced morphological damage at 31·5°C, the plants merely being much smaller than those grown at 25°C. The characteristic phenotypes of the other lesion races at 31·5°C are described below. Their appearances were contrasted with plants grown at 25°C where they have the following approximate dimensions: hypocotyl, 2 mm long; first leaf pair, 3·0 mm diam.; second leaf pair, 2·3 mm; third leaf, 2·0 mm; fourth leaf, 0·8 mm.

TABLE 4

ARITHMETIC FRESH WEIGHT AND LOG FRESH WEIGHT MEANS (IN MG) FOR FIVE HIGH-TEMPERATURE-SENSITIVE RACES ON A RANGE OF ORGANIC SUPPLEMENTS AT 31·5°C

Race	Control		Casein Hydrolysate (0·3 mg)		Nucleic Acids (0·3 mg)		Yeast Extract (0·2 mg)		Vitamins (0·05 ml)	
	Arith.	Log	Arith.	Log	Arith.	Log	Arith.	Log	Arith.	Log
BLA	3·06	0·464	3·86	0·574	3·44	0·518	2·46	0·377	4·26	0·614*
DI	4·55	0·598	4·30	0·594	4·44	0·600	3·35	0·487	4·99	0·659
HI	5·13	0·692	4·05	0·594	4·74	0·618	2·87	0·414	2·17	0·291
LS	9·05	0·931	10·39	0·962	12·64	1·076*	10·24	0·985	8·57	0·876
PI	4·02	0·568	3·50	0·508	4·33	0·605	5·27	0·689*	2·91	0·433

*Indicates significantly greater weight than control (see text).

PI.—Lesion evident in an extreme elongation of the hypocotyl (16 mm) and a breakdown in leaf growth. Although the cotyledons are normal, the first leaf pair is very small (0·7 mm diam.) with narrow laminae and short thin petioles; in place of the second leaf pair are rudimentary undifferentiated structures (0·3 mm long); subsequent leaves are absent.

BLA.—Lesion similar to that of PI, but there is no abnormal hypocotyl elongation and leaf growth is more suppressed. The first leaf pair (0·2 mm diam.) is almost devoid of laminae and is whitish about the edges; subsequent leaves are absent.

DI.—Plants small, weak, and chlorotic. The hypocotyl is very elongated (12 mm) and leaf diameter reduced; first leaf pair, 2·2 mm; second leaf pair, 1·8 mm; third leaf, 0·9 mm.

HI.—Leaves very small and curled downwards; first leaf pair, 2·5 mm diam.; second leaf pair, 1·2 mm; third leaf, 0·6 mm.

LS.—Leaf and root growth quite good, but the third and fourth leaves contain chlorotic patches, and the flower stalks are characteristically curled and twisted.

(*c*) *Experiment* 3

On the basis of the data from experiment 2, five races (PI, BLA, DI, HI, and LS) with greatly reduced growth and pronounced deficiency symptoms at 31·5°C were grown at this temperature on a full range of organic supplements. These races were supplied with yeast extract (0·2 mg/plant), casein hydrolysate (0·3 mg), nucleic acid hydrolysate (0·3 mg), and vitamins (0·05 ml). The methods for preparing the supplements and media have been described previously (Langridge 1958). Twenty seeds were planted for each race–supplement combination, but unequal germination reduced the mean number of plants per treatment to approximately 17. The fresh weight and log fresh weight means after 14 days' growth are given in Table 4.

TABLE 5

ARITHMETIC FRESH WEIGHT AND LOG FRESH WEIGHT MEANS (IN MG) FOR THREE HIGH-TEMPERATURE-SENSITIVE RACES ON SELECTED SUPPLEMENTS AT TWO TEMPERATURES

Race	Temp. (°C)	Yeast Extract (mg)							
		0·0		0·1		0·2		0·3	
		Arith.	Log	Arith.	Log	Arith.	Log	Arith.	Log
PI	25	37·23	1·556	34·94	1·537	32·08	1·499	29·64	1·467
	30	6·74	0·798	7·05	0·832	6·61	0·807	5·44	0·724
		Nucleic Acids (mg)							
		0·0		0·15		0·30		0·45	
		Arith.	Log	Arith.	Log	Arith.	Log	Arith.	Log
LS	25	31·84	1·498	24·13	1·381	23·56	1·365	20·78	1·311
	30	9·37	0·939	10·29	0·998	11·45	1·036	11·66	1·051
		Vitamins (ml)							
		0·0		0·1					
		Arith.	Log	Arith.	Log				
BLA	25	30·25	1·480	26·66	1·420				
	30	7·17	0·846	8·02	0·891				

This experiment was designed to indicate which, if any, of the supplements stimulate growth beyond that recorded by the control. A comparison by the usual analysis of variance indicated that significant increases in log fresh weight had occurred in three of the races.

Race	Supplement	Probability Associated with F Ratio
BLA	Vitamins	$P<0\cdot005$
LS	Nucleic acids	$0\cdot010<P<0\cdot025$
PI	Yeast extract	$0\cdot025<P<0\cdot050$

The two other races, DI and HI, gave no significant stimulatory effects.

Although these results are interesting in indicating a partial breakdown in the characteristic autotrophy of green flowering plants, they are not proof of the occurrence of "preferential or specific high temperature stimulatory responses".

TABLE 6

ANALYSIS OF VARIANCE INVOLVING THE FIRST LEVEL OF SUPPLEMENTS ONLY FOR THE RACES PI, LS, AND BLA

Source	PI		LS		BLA	
	Yeast Extract (0·10 mg)		Nucleic Acids (0·15 mg)		Vitamins (0·10 ml)	
	D.F.	Mean Squares	D.F.	Mean Squares	D.F.	Mean Squares
Temperature	1	10·012,896**	1	3·259,272**	1	4·897,984**
Supplement	1	0·000,990	1	0·012,409	1	0·000,808
Interaction	1	0·012,821	1	0·113,937**	1	0·040,001*
Error	71	0·017,680	56	0·011,997	54	0·007,582

$*0\cdot01<P<0\cdot05$. $**P<0\cdot01$.

(*d*) *Experiment* 4

As explained above, the hypothesis requires that the supplement produces a differential response at optimal and high growth temperatures so as to fulfil the requirements for a preferential or specific stimulatory response. The responding races were therefore grown with and without the supplement at two temperatures.

It seemed possible that the relatively poor supplement response obtained in experiment 3 might have resulted from (1) the supplement concentration being non-optimal, or (2) the temperature being so high that, in addition to an inactivation of a presumed major growth reaction, a number of other physiological processes had also been damaged. Accordingly, a concentration series of the appropriate supplement was supplied to two of the races (PI and LS), while the temperature was reduced to 30°C to lessen the probability of multiple inhibitions. The treated races, growth temperatures, and supplement type and concentrations are all entered in Table 5, which contains the resultant fresh weight and log fresh weight mean values after 14 days' growth.

From the growth data for PI, it is clear that the slight stimulus with the first level of yeast extract disappears with succeeding levels. With LS, however, growth stimulation is increased by higher concentrations of nucleic acid hydrolysate. This stimulatory effect is of a higher order of magnitude than that found for PI.

Factorial analyses of variance, which involve the lowest level of supplements only, are given in Table 6. *F* ratios involving the interaction mean square provide a test for the "specific high temperature stimulatory response". The analyses of variance were computed with disproportionate numbers in the usual way. The following results were found with respect to the test for interaction:

Race	Supplement	Probability Associated with *F* Ratio
PI	Yeast extract	Not significant
LS	Nucleic acids	$P<0\cdot005$
BLA	Vitamins	$0\cdot010<P<0\cdot050$

The analysis indicates that one or more components of the nucleic acid hydrolysate possesses stimulatory activity with respect to the growth of LS at high temperature, and one or more vitamins possesses similar activity with respect to the race BLA.

To determine the specific stimulatory component of nucleic acid hydrolysate, plants of LS were grown for 14 days with the ribosides of the four nucleic acid bases added to the medium (0·25 mg riboside per plant). To discriminate between the 10 water-soluble vitamins in the vitamin mixture, plants of BLA were grown with the addition of the following four solutions (0·1 ml per plant):

Solution I:	riboflavin, nicotinic acid, pantothenic acid, pyridoxine.
Solution II:	nicotinic acid, biotin, inositol, folic acid.
Solution III:	pantothenic acid, inositol, ascorbic acid, choline.
Solution IV:	pyridoxine, folic acid, choline, *p*-aminobenzoic acid.

In the absence of multiple requirements, these four treatments are capable of giving 10 possible types of response, one for each vitamin (Lindegren and Lindegren 1951).

The resultant fresh weights (Table 7) show that for the growth of LS at high temperature, cytidine is the active stimulatory component of nucleic acid hydrolysate. In the case of BLA, growth on solution II indicates that biotin is very probably the vitamin required by this race.

As a confirmatory test, BLA was grown with and without biotin (3 μg/plant) at 25 and 31°C for a period of 21 days. The arithmetic fresh weight means for plants with and without biotin at 25°C were 42·78 and 36·07 mg respectively, and the means for plants with and without biotin at 31°C were 14·89 and 5·05 mg respectively. Thus, biotin was 2·5 times as effective at the high as at the optimal

TABLE 7

ARITHMETIC FRESH WEIGHT AND LOG FRESH WEIGHT MEANS (IN MG) FOR THE TEMPERATURE-SENSITIVE RACES LS, BLA, AND PI ON VARIOUS ORGANIC SUPPLEMENTS AT 31°C

Number of observations are given in parenthesis after the log means

Race	Control		Uridine		Cytidine		Adenosine		Guanosine	
	Arith.	Log	Arith.	Log	Arith.	Log	Arith.	Log	Arith.	Log
LS	12·31	1·047 (17) ±0·080	13·27	1·113 (16) ±0·024	15·39	1·174* (23) ±0·023	13·09	1·080 (16) ±0·049	8·95	0·947 (15) ±0·017
	Control		Vitamin Solution							
			I		II		III		IV	
	Arith.	Log	Arith.	Log	Arith.	Log	Arith.	Log	Arith.	Log
BLA	4·75	0·672 (15) ±0·018	3·07	0·466 (18) ±0·033	6·19	0·766** (14) ±0·044	1·42	0·079 (6) ±0·114	3·55	0·509 (12) ±0·061
PI	6·01	0·764 (13) ±0·034	2·45	0·348 (11) ±0·063	8·68	0·905* (13) ±0·049	4·82	0·658 (12) ±0·043	4·80	0·655 (13) ±0·043

*Significantly greater weight than control with $0{\cdot}025 < P < 0{\cdot}050$.

**Significantly greater weight than control with $P \cong 0{\cdot}05$.

temperature. An analysis of variance of the logarithmic values yielded a highly significant interaction effect ($P \ll 0 \cdot 005$). In addition to significantly increasing the growth of the plants at the high temperature, biotin removed essentially all of the characteristic symptoms of high temperature damage in BLA (see Plate 1).

The striking effect of biotin on the leaf growth of BLA when supplied alone or in vitamin solution II was not apparent when supplied in the complete vitamin mixture. Biotin is evidently antagonized by some other vitamin or vitamins in the mixture. In view of such strong antagonisms, the requirement of the race PI, which previously gave a slight response to yeast extract, was re-investigated. The supplements (vitamins, amino acids, and nucleic acid bases) were supplied as mixtures each having relatively few components. After 21 days' growth at 31°C, the fresh weight of plants of PI on vitamin solution II (composition as above) was significantly greater than the control (Table 7). All other supplements were without growth-

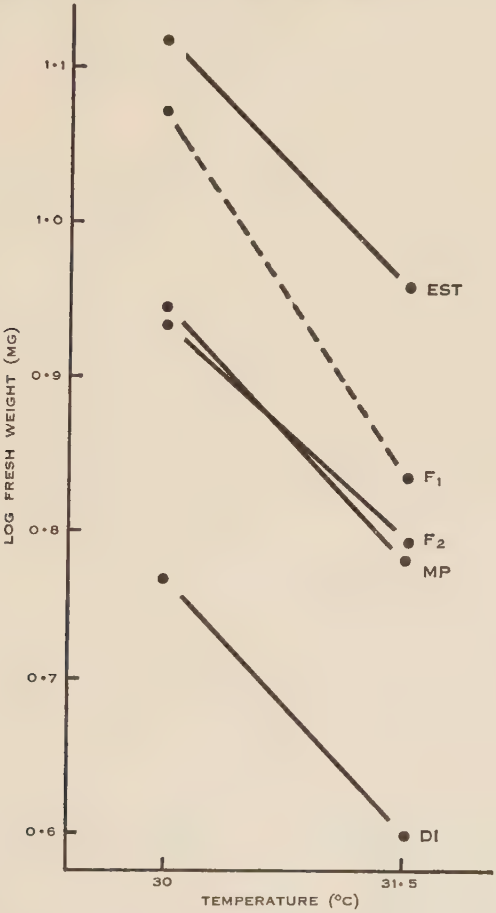


Fig. 5.—Log fresh weight means plotted against two temperatures for EST, DI, F_1 (EST × DI), F_2 (EST × DI), and the mid-parent (MP).

promoting effect. Growth stimulation on only vitamin solution II indicates that biotin is again the active component. This conclusion was immediately tested by the aseptic addition of sterile biotin solution (6 μg/plant) to several plants of the control. These treated plants formed several pairs of large leaves and then flower stalks, whereas the control plants ceased growth after the first leaf pair. Parenthetically, it may be noted that these two races, BLA and PI, although they both require biotin at high temperatures, have been collected from wild populations about 800 miles apart. Races of intermediate geographical origin, e.g. TU (Turin), appear not to have the requirement. This suggests that the genic differences causing the requirement may have arisen independently.

(*e*) *Experiment* 5

Some F_1 and F_2 stocks, which had been prepared for another purpose, were examined in order to obtain some indication of the genetic basis of the high temperature sensitivity phenomenon.

The log fresh weights of parents, F_1's, and F_2's of the cross EST × DI at 30 and 31·5°C are set out in Figure 5. The growth of the F_1 relative to its parents is quite different at the two temperatures. At 30°C the F_1 log mean is close to that of the high parent, but at 31·5°C the F_1 log mean is close to the mid-parental value. This may indicate that different sets of genes with different dominance properties are brought into play at the different temperatures. However, it should be pointed out that the F_2 mean for 30°C is slightly below the mid-parental value. This is a surprisingly low value for the F_2 on the basis of nearly complete dominance of plus genes as indicated by the F_1. It is clear that the change of dominance with temperature needs to be more thoroughly investigated.

In a preliminary examination of some of the F_1's between temperature-sensitive and non-temperature-sensitive races, there appeared a considerable range of F_1 expression relative to the parental values. F_1's were obtained varying from those showing no dominance to those exhibiting overdominance.

V. Discussion

The demonstration that temperatures above the growth optimum are more damaging to plants than those below the optimum was not unexpected. While suboptimal temperatures reduce the rate of chemical reactions contributing to growth, supra-optimal temperatures permit the incidence, and accelerate the rate, of destructive reactions. Such destructive processes, e.g. enzyme denaturation, have higher temperature coefficients than ordinary catalytic reactions, and thus there is a sharp decrease in growth as the temperature increases above the optimum. It is presumably a differential denaturation of particular enzymes as the growth temperature is raised that causes the steady increase in the genotypic variance component. The existence in the tested collection of several races very sensitive to higher temperatures may mean that these races possess one or more genes controlling enzymes of more than average lability to temperature.

Thus far, the first part of the hypothesis we are testing, viz. that some races are especially predisposed to high temperature damage, has proved correct. It remains to consider the second part of the hypothesis, first presented by Bonner (1957), viz. that the temperature-induced deficiencies may be repaired by the application of appropriate chemicals.

If every essential genetic function possessed an equal probability of being inactivated by high temperature, the resultant temperature lesions would usually not be reparable. The inactivations would affect metabolites which are unable to diffuse through membranes or which are very unstable, as well as stable, diffusible substances. The former class is very frequent as indicated by the work of Lein, Mitchell, and Houlahan (1948), who found that only 7 per cent. of growth mutants in *Neurospora* responded to an autolysate of the fungus.

TABLE 8

FREQUENCIES OF CERTAIN TYPES OF GROWTH MUTANTS IN ESCHERICHIA, NEUROSPORA, AND ARABIDOPSIS

— = not measured

Organism	Frequency of Biochemical Mutants				Reference
	Temperature Sensitive		Non-temperature Sensitive		
	Reparable	Non-reparable	Reparable	Non-reparable	
Escherichia coli	124 (77%)	37 (23%)	—	—	Leupold and Horowitz (1951)
Neurospora crassa	14 (54%)	12 (46%)	—	—	Horowitz (1950)
			2 (8%)	24 (92%)	Atwood and Mukai (1953)
Arabidopsis thaliana	6 (75%)	2 (25%)	2 (29%)	5 (71%)	Langridge (1958) and unpublished data

However, with respect to temperature, there seem to be two classes of gene function. In the first and much larger class, neither the immediate nor subsequent products of gene action appear to be replaceable from external sources or able to exist in temperature-labile forms. Gene products of the second class may be temperature labile and either they, or the metabolites they control, can often be absorbed by the organism from the substrate. The data from which these contentions are derived are shown in Table 8 together with the few data available for *A. thaliana*.

The frequency of non-reparable, non-temperature-sensitive mutants in *Escherichia* is difficult to obtain, and has not been published. It is known, however, that only a small percentage of mutants with reduced growth are reparable, in contrast with the high frequency of reparability in the temperature-sensitive class.

In *Neurospora*, it is apparent, as has been noted previously by Atwood and Mukai (1953), that a mutant which is found to be temperature sensitive is many times more likely to require a diffusible compound than a mutant insensitive to temperature. This classification is not related to the type of synthesis affected. Horowitz (1950) and Leupold and Horowitz (1951) have shown that temperature-sensitive mutations occur at random among the genes affecting known biochemical reactions.

Assuming that the general metabolism of a flowering plant is essentially similar to that of a fungus, one may expect many of the high temperature lesion races of *Arabidopsis* to respond to supplements. The data so far obtained with this plant are too few, however, to test the theory.

Because these races with a reparable high temperature lesion require only single compounds for normal growth, it is assumed that growth inactivation results from the loss in activity of single genes. A molecular model for such gene–temperature interactions is provided by the work of Maas and Davis (1952) and Horowitz and Fling (1953). They examined two mutants, in *Escherichia* and *Neurospora* respectively, which exhibited requirements for metabolites only at high temperatures. Both mutants were found to possess mutant alleles controlling enzymes more rapidly inactivated by temperature than the enzymes produced by the corresponding wild-type alleles.

Of the five temperature-lesion races which have been studied, only three showed a response to the supplements supplied. It is possible that the metabolites required by the non-responding races were not present in the supplements, although yeast extract might be expected to contain most of the diffusible substances needed by plants. A more serious deficiency in the method, however, results from the supplying of metabolites in mixtures. Antagonism between the components of such mixtures is very common; for example, biotin in the presence of the nine other vitamins of the complete mixture has no apparent effect on the leaf growth of BLA. Therefore, a lack of response to such a mixture does not necessarily mean that the required metabolite is not present in the mixture.

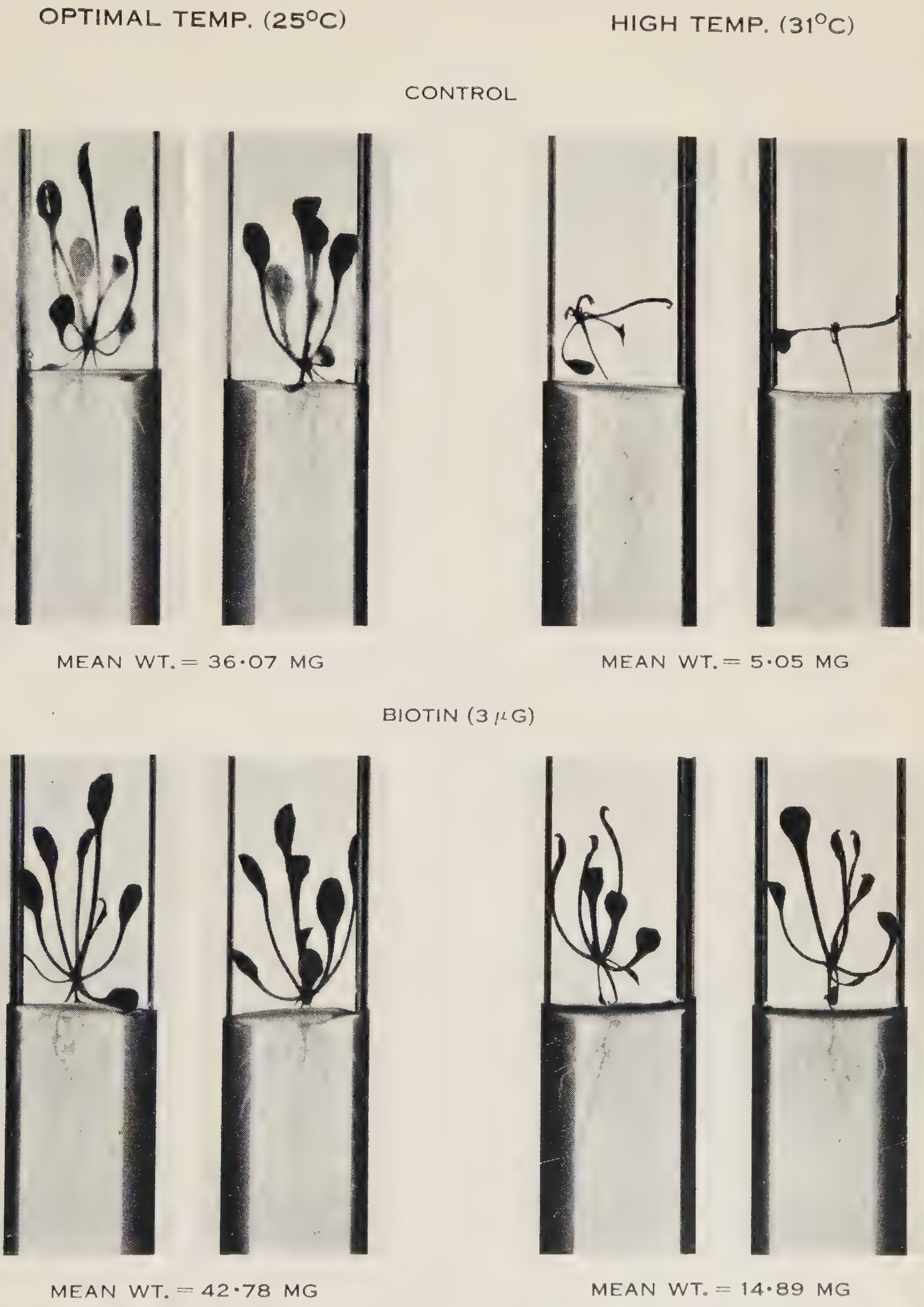
Our results show that some wild populations of *A. thaliana* possess genes, in some cases probably single genes, whose products become inactive at high temperatures where the consequent deficiencies severely limit plant growth. It is possible that similar genes may be quite widespread amongst cultivated plants adapted to temperate climates, and the presence of such genes may be one of the factors which prevents their introduction into warmer or subtropical regions.

The most simple and economical way to overcome the growth limitations imposed by the presence of a temperature-sensitive gene in a cultivated plant would be to replace it by its temperature-resistant allele. This may not always be possible. especially in cases where there is no genetically available source of such a gene. Then recourse must be had to an artificial mutation programme, or the direct application of the missing metabolite to the crop.

VI. ACKNOWLEDGMENTS

We would like to acknowledge the competent assistance of Miss Roma Brown and Miss Helen Haylock. We are also grateful for suggestions by Mr. G. Wilkinson.

HIGH TEMPERATURE LESIONS IN ARABIDOPSIS



Pictorial representation of the temperature–biotin interactions of the race BLA.

VII. REFERENCES

ATWOOD, K. C., and MUKAI, F. (1953).—Indispensable gene functions in *Neurospora*. *Proc. Nat. Acad. Sci., Wash.* **39**: 1027–35.

BONNER, J. (1943).—Effects of application of thiamine to *Cosmos*. *Bot. Gaz.* **104**: 475–9.

BONNER, J. (1957).—The chemical cure of climatic lesions. *Engng. Sci. Mag.* **20**: 28–30.

GALSTON, A. W. (1957).—In "The Experimental Control of Plant Growth". (Ed. F. W. Went.) pp. 313–17. (Chronica Botanica Co.: Waltham, Mass.)

GALSTON, A. W., and HAND, M. E. (1949).—Adenine as a growth factor for etiolated peas and its relation to the thermal inactivation of growth. *Arch. Biochem.* **22**: 434–43.

HOROWITZ, N. H. (1950).—Biochemical genetics of *Neurospora*. *Advances in Genetics* **3**: 33–71.

HOROWITZ, N. H., and FLING, M. (1953).—Genetic determination of tyrosinase thermostability in *Neurospora*. *Genetics* **38**: 360–74.

LANGRIDGE, J. (1957).—The aseptic culture of *Arabidopsis thaliana* (L.) Heynh. *Aust. J. Biol. Sci.* **10**: 243–52.

LANGRIDGE, J. (1958).—A hypothesis of developmental selection exemplified by lethal and semi-lethal mutants of *Arabidopsis*. *Aust. J. Biol. Sci.* **11**: 58–68.

LEIN, J., MITCHELL, H. K., and HOULAHAN, M. B. (1948).—A method of selection of biochemical mutants of *Neurospora*. *Proc. Nat. Acad. Sci., Wash.* **34**: 435–42.

LEUPOLD, U., and HOROWITZ, N. H. (1951).—Über Temperaturmutanten bei *Escherichia coli* und ihre Bedeutung für die "Ein Gen–Ein Enzym" Hypothese. *Z. indukt. Abstamm.-u. Vererb-Lehre* **84**: 306–19.

LINDEGREN, C. C., and LINDEGREN, G. (1951).—Linkage relations in *Saccharomyces* of genes controlling the formation of carbohydrates and the synthesis of vitamins, amino-acids and nucleic acid components. *Indian Phytopath.* **4**: 11–20.

MAAS, W. K., and DAVIS, B. D. (1952).—Production of an altered pantothenate-synthesizing enzyme by a temperature-sensitive mutant of *Escherichia coli*. *Proc. Nat. Acad. Sci., Wash.* **38**: 785–97.

THE WATER PERMEABILITY OF CELLS OF *CHARA AUSTRALIS* R.BR.

By J. Dainty* and A. B. Hope†

[*Manuscript received October* 31, 1958]

Summary

Measurements have been made of the "osmotic" water permeability (P_{os}) of internodal cells of *Chara australis* using the method of transcellular osmosis. The mean value was $5 \cdot 6\ \mu^3/\mu^2 . \text{min} . \text{atm}$, which is close to that obtained by Kamiya and Tazawa (1956) with cells of *Nitella.* A small difference between rates of water movement into and out of the cell is ascribed to an assymetry of the driving forces in the system and not to a real difference in permeability.

Attempts to measure the "diffusional" water permeability (P_d) of similar cells by studying the isotopic exchange of D_2O for the cell H_2O were not successful for reasons discussed.

The basis for comparison of P_{os} with P_d is discussed with particular reference to concepts of "filtration" and "bulk flow" of water through pores in the cell membrane.

I. Introduction

The water permeability of plant cells has, until recently, been studied by methods involving plasmolysis and deplasmolysis. The experiments of Osterhout (1949*a*, 1949*b*) and the important paper of Kamiya and Tazawa (1956) have introduced a new method—transcellular osmosis—which is applicable to large cylindrical cells and which does not involve plasmolysis. In this paper the application of this method to the study of the "osmotic" water permeability (P_{os}) of the large, single internodal cells of *Chara australis* R.Br. var. *nobilis* is described; we have also tried to measure with the same cells the "diffusional" water permeability (P_d) by studying isotopic exchange of the cell water.

Both these permeabilities have been studied separately on cells from members of the Characeae, the osmotic permeability of *Tolypellopsis* (now named *Nitellopsis*) by Palva (1939) and of *Nitella* by Kamiya and Tazawa (1956), and the diffusional permeability of *Tolypellopsis* by Wartiowaara (1944). A comparison of the P_{os} and P_d values of several different animal cells (amoeba, various fish and amphibian eggs (Prescott and Zeuthen 1953); red blood cells (Paganelli and Solomon 1957)) has been used to make deductions about the size and number of pores in the cell membrane.

In the present experiments we have obtained a value for the osmotic permeability of internodal cells of *Chara australis*, but attempts to measure diffusional permeability failed for reasons set out in Section V. However, the merits of a comparison between P_{os} and P_d are discussed because it seems to us that a number of

---

* Biophysics Department, University of Edinburgh.

† Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., and Botany School, University of Sydney.

misconceptions about the meanings of osmotic and diffusional permeability are current.

II. Theory of Transcellular Osmosis

In the theories of transcellular osmosis which have already been published, cf. a simple version by Osterhout (1949*a*, 1949*b*) and a detailed theory by Kamiya and Tazawa (1956), the role of the changes in turgor pressure has not been considered and hence, in certain respects, these theories are inadequate. In the following paragraphs a simple account of transcellular osmosis is given which is believed to be a more correct description of the phenomenon than previous accounts. Certain assumptions have, however, been made. These are that the cell is perfectly semi-permeable, i.e. the permeability to water is infinitely greater than that to solutes such as sucrose and the ions in the cell vacuole. This in turn assumes no active mechanisms involving water transport and that the ion transport mechanism(s) contribute negligible fluxes compared with the fluxes of water. These assumptions are probably well founded (Mercer 1955; MacRobbie and Dainty 1958). The movement of water along the cell wall is assumed to be negligible (see Kamiya and Tazawa 1956). The experimental arrangement is shown diagrammatically in Figure 1, where the symbols used are also given.

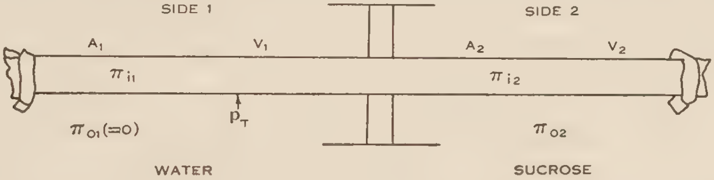


Fig. 1.—The situation during transcellular osmosis which is discussed in Section II. π_{i1} and π_{i2} are internal osmotic pressures due to vacuolar solutes on side 1 or 2. π_{01} and π_{02} are external osmotic pressures (zero on side 1 which contains water). p_T is the turgor pressure, constant along the length of the cell.

When distilled water is bathing both ends of the cell, there will be no net suction tension and the cell will have a turgor pressure, p_T, equal to π, the initial osmotic pressure of the sap solution. At $t = 0$, the water at end 2 of the cell is replaced by a sucrose solution of osmotic pressure π_{02} atm (the time necessary to change the solutions and the time for the sucrose to diffuse to the surface of the cell are neglected). Then, at $t = 0$, the suction tension at end 1 is still zero, but at end 2 it is equal to $\pi_{02}-(\pi-p_T) = \pi_{02}$. Thus water starts to move out from the cell at end 2 at a rate given by $P_{os} . A_2 . \pi_{02}$,* where P_{os} is the osmotic permeability of the cell membrane in cm sec^{-1} atm^{-1} and A_2 the cell area at end 2. There is no initial movement of water into end 1 of the cell.

The effect of a movement of Δv c.c. of water out of end 2 is to decrease the volume of water in the cell and hence to reduce the turgor pressure of the cell by

---

* Our results indicate some difference between the rates of water movement into and out of the cell, but we shall assume the same permeability coefficient P_{os} for both endosmosis and exosmosis for reasons given in Section V.

Δp_T. The volume of water in the compartment around end 1 would *apparently* decrease because of cell shrinkage and indicate an *apparent* movement of water through the cell equal to $\Delta v . V_1/V$, where V_1 is the cell volume in end 1 and V the total cell volume. The decrease Δp_T in turgor pressure would start a *real* flow of water into end 1 given by $P_{os} . A_1[\pi-(p_T-\Delta p_T)]$. This would be small compared with the flow of end 2 if the cell were very extensible, i.e. if $\Delta p_T/\Delta v$ were small; in this case the apparent initial rate of flow of water through the cell would be given by $P_{os} . A_2 . \pi_{02} . V_1/V$, which equals $P_{os} . A_1 A_2 . \pi_{02}/A$. The cells of *Chara australis* are not very extensible; an outflow of a few tenths of a microlitre produces a change in turgor pressure of 7 or 8 atm. Thus a net outflow of a fraction of a microlitre will produce such a large drop in turgor pressure that an appreciable inflow of water almost immediately occurs into end 1 and the steady state in which

$$P_{os} . A_1(\pi_{i1}-p_T) = P_{os} . A_2[\pi_{02}-(\pi_{i2}-p_T)], \quad \ldots\ldots(1)$$

is reached after a net outflow of a few tenths of a microlitre.

π_{i1}, π_{i2} are the osmotic pressures in the cell on sides 1 and 2 respectively.

It is clear from equation (1) that when the steady state has been reached, the turgor pressure of the cell has decreased to

$$\begin{aligned} p_T &= (A_1\,\pi_{i1}+A_2\,\pi_{i2})/(A_1+A_2)-A_2\,\pi_{02}/(A_1+A_2) \\ &= \pi - A_2\,\pi_{02}/A. \quad \ldots\ldots\ldots\ldots\ldots\ldots(2) \end{aligned}$$

We can write for the steady state rate of flow, dv/dt, the following equations:

$$\mathrm{d}v/\mathrm{d}t = P_{os} . A_1(\pi_{i1}-p_T), \quad \ldots\ldots\ldots\ldots\ldots\ldots(3)$$

$$\mathrm{d}v/\mathrm{d}t = P_{os} . A_2[\pi_{02}-(\pi_{i2}-p_T)], \quad \ldots\ldots\ldots\ldots\ldots\ldots(4)$$

and these two equations can be combined to one equation:

$$\mathrm{d}v/\mathrm{d}t = K(\pi_{i1}-\pi_{i2}+\pi_{02}), \quad \ldots\ldots\ldots\ldots\ldots\ldots(5)$$

where

$$K = P_{os} . \frac{A_1 A_2}{A_1+A_2} \quad \text{or} \quad P_{os}\Big/\left(\frac{1}{A_1}+\frac{1}{A_2}\right). \quad \ldots\ldots(6)$$

From equation (5) Kamiya and Tazawa (1956) have developed a detailed theory of transcellular osmosis. Qualitatively, water flows into end 1 and then moves along to end 2, inside the cell, carrying with it a proportion of the internal solutes; pure water leaves at end 2. Thus the water flow results in an increasing polarization of the sap solution; it becomes more concentrated at the sucrose end (end 2) and more dilute at the water end (end 1). This polarization has been directly determined by Kamiya and Kuroda (1956). As can be seen from equation (5) polarization of the internal solutes decreases the rate of flow of water which would cease when polarization had produced zero suction tensions at the two ends of the cell. This final state is never quite reached because the polarization is opposed by solute diffusion and by stirring of the sap by the protoplasmic streaming.

Kamiya and Tazawa (1956) give a quantitative theory of the events described above which fits their results reasonably well. However, the theory contains certain simplifying assumptions and does not take into account the complicated geometry of the system. We have, therefore, thought it preferable to use only the initial rates of water movement as a measure of permeability. This initial rate is given by

$$\mathrm{d}v/\mathrm{d}t = K\pi_{02}, \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(7)$$

hence

$$P_{os} = K\left(\frac{1}{A_1}+\frac{1}{A_2}\right) = \frac{\mathrm{d}v/\mathrm{d}t}{\pi_{02}}\left(\frac{1}{A_1}+\frac{1}{A_2}\right). \qquad \ldots\ldots\ldots\ldots(8)$$

Equation (7) contains no doubtful theoretical assumptions, but it is necessary to ensure that there is no appreciable polarization of the internal solutes during the period of measurement.

III. Experimental Methods

The osmotic permeability was calculated from the rate of water movement through a measured area of cell surface under a known osmotic gradient, using equation (8), the rate being measured during transcellular osmosis. The apparatus used for most of the experiments was similar to that of Osterhout (1949*a*, 1949*b*) and is illustrated in Figure 2. The apparatus and flasks of solution were immersed in a water-bath at 25±0·01°C. The condition of the cells, as judged by the protoplasmic streaming, could be checked at any time with the aid of a binocular microscope. The cells were not damaged by overall osmotic gradients of up to 14 atm; at this value occasional "splitting" of the ordered chloroplast layers was observed. Sucrose solutions were used to produce the osmotic pressure gradients. One centimetre travel of the air bubble in the capillary corresponded to a transfer of 0·912 μl of water.

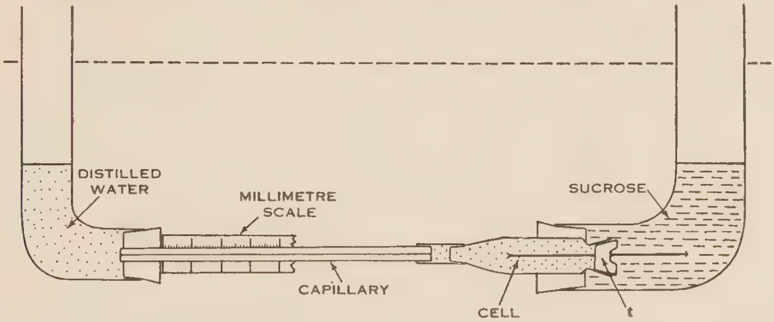


Fig. 2.—Form of the apparatus used to measure the rate of water movement during transcellular osmosis. It is very similar to that of Osterhout (1949*a*, 1949*b*). *t*, Split "Perspex" taper with a central 1·6 mm diameter hole in which the cell was sealed with "Vaseline".

The rate of exchange of D_2O with H_2O was measured from the change in reduced weight with time after the cell was transferred from a D_2O–H_2O mixture, in which it had equilibrated, to a large volume of water. This method is analogous to that of Løvtrup and Pigón (1951). Similar experiments for comparison were done with agar cylinders of the same dimensions as the cells.

IV. RESULTS

(*a*) *Equal Cell Areas in Sucrose and Water*

Figures 3 and 4 show results from a typical cell. In Table 1 the results from three different cells for various osmotic pressure gradients are collected. P_{os} was calculated from equation (8). During the initial few seconds, the rate of flow was low because of the time required, on changing from water to sucrose solution, for diffusion of the sucrose solution to the surface of the cell. Therefore, in calculating the initial rate of flow from the initial slope of the curve of volume transferred *v.* time, the initial point and sometimes the second point was ignored.

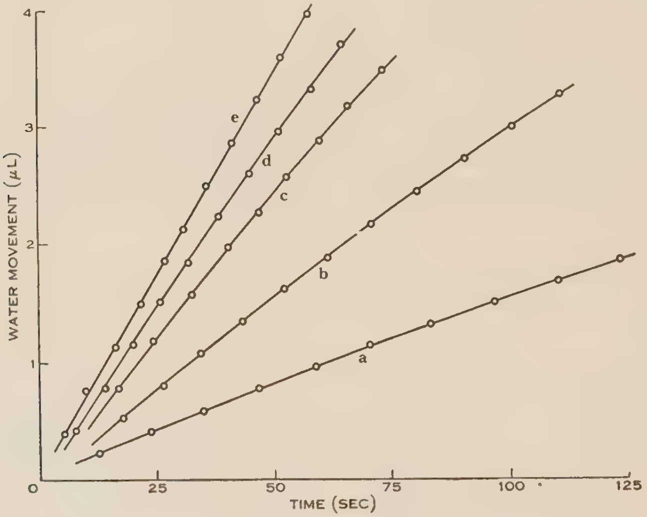


Fig. 3.—Volume of water transferred during transcellular osmosis (in microlitres) plotted against time (in seconds). In curves *a–e*, the osmotic gradients between sides 1 and 2 were due to 0·1, 0·2, 0·3, 0·4, and 0·5M sucrose respectively being placed on side 2. The areas on sides 1 and 2 were equal.

The mean value of P_{os} from these experiments is $5\cdot6\ \mu^3/\mu^2$. min . atm. If osmotic pressure and volume of water are expressed in moles/cm^3 and moles respectively and the time in seconds, P_{os} can be expressed in more general units, i.e. 123×10^{-4} cm/sec or 123 μ/sec.

(*b*) *Unequal Cell Areas in Sucrose and Water*

Kamiya and Tazawa (1956) found unequal rates of water transfer through the cell according to whether the larger area of the cell was in water or sucrose. This was interpreted as a difference in P_{os} for water movement into and out of the cell. In their smaller cells, appreciable internal solute polarization is produced by quite a small water transfer, hence it is difficult to measure the initial rate of flow accurately. It was therefore decided to repeat their experiments, using a very asymmetrical arrangement, on *Chara australis*, where the polarization produced by a given transfer of water is much smaller.

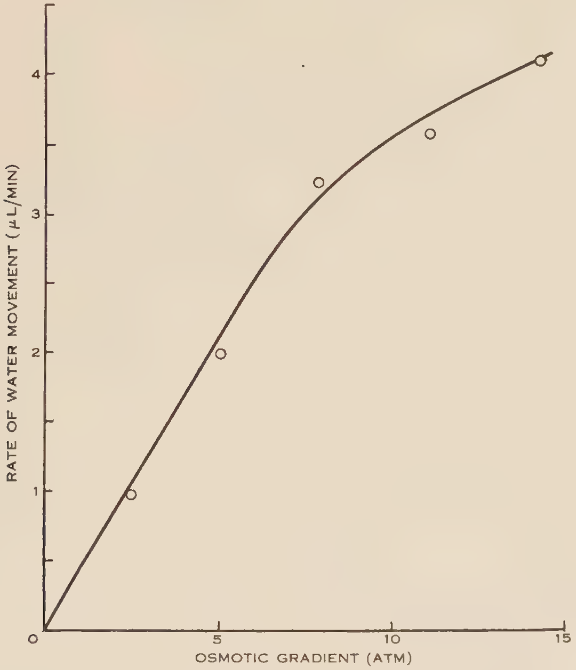


Fig. 4.—Relation between rate of water transfer (in μl/min) and the osmotic gradient (in atm). Data from cell 3, Table 1.

TABLE 1

OSMOTIC PERMEABILITIES OF CHARA AUSTRALIS CELLS

Sucrose Concn. (M)	Osmotic Pressure (atm)	Osmotic Permeabilities (μ^3/μ^2 . min . atm)		
		Cell 1	Cell 2	Cell 3
0·05	1·25	—	5·2	—
0·1	2·5	4·7	6·1	6·2
0·2	5·0	4·2	5·6	6·3
0·3	7·8	—	5·2	6·6
0·35	9·3	3·6	—	—
0·4	11·0	—	4·5	5·2
0·5	14·2	3·7	4·4	4·6

The water transfer during transcellular osmosis in a cell with first the larger area in water and the smaller area in 0·2M sucrose and then vice versa is shown in

Figure 5. It can be seen that the rates were in the ratio of about 1 : 1·2 when the ratios of the areas was 5 : 1. This and six other measurements led to an apparent ratio of the osmotic permeabilities in the inward and outward directions of 1·4 ± 0·1,* when the sucrose concentration was 0·2M.

V. DISCUSSION

(a) *The Osmotic Permeability*

The present experiments with *Chara australis* cells lead to a mean value of 5·6 μ^3/μ^2 . min . atm or 123 μ/sec for P_{os}. This is similar to the value obtained by Kamiya and Tazawa (1956) for *Nitella flexilis*, i.e. 7–18 μ^3/μ^2 . min . atm. The value of P_{os} obtained by Palva (1939) for *Tolypellopsis* was 24 μ/sec. This was obtained from measurements of the rate of change of the reduced weight of a cell when osmotic water movement was taking place and is not likely to be very accurate.

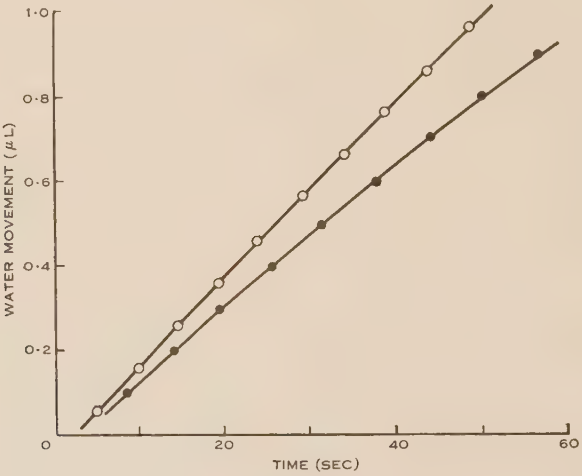


Fig. 5.—Volume of water transferred (in microlitres) plotted against time (in seconds) when the areas of the cell in compartments 1 and 2 were unequal. ● Sucrose (0·2M) at the shorter end. ○ Sucrose (0·2M) at the longer end. The ratio of the areas was 5 : 1.

---

* If P'_{os} is the permeability for inward water movement and P''_{os} is the permeability for outward water movement it can easily be seen from appropriate modifications of equations (3) and (4) that

$$\frac{P'_{os}}{P''_{os}} = \frac{\dfrac{A_1}{A_2} - \dfrac{(\mathrm{d}v/\mathrm{d}t)_1}{(\mathrm{d}v/\mathrm{d}t)_2}}{\left(\dfrac{A_1}{A_2}\right)\dfrac{(\mathrm{d}v/\mathrm{d}t)_1}{(\mathrm{d}v/\mathrm{d}t)_2} - 1}, \qquad \text{.....................(9)}$$

where A_1/A_2 is the ratio of the areas and $(\mathrm{d}v/\mathrm{d}t)_1/(\mathrm{d}v/\mathrm{d}t)_2$ is the ratio of initial rates of water flow in the two cases outlined above.

From the experiments with very unequal areas of the cell in sucrose and water, it would appear that the permeability to water moving into the cell is $1 \cdot 4 \pm 0 \cdot 1$ times greater than the permeability to water moving out of the cell. Kamiya and Tazawa (1956) found a value of 2·66 for the ratio of these two permeabilities, but inspection of their Figure 8 suggests that the rapid polarization of sap solutes led them to overestimate this ratio. They consider this difference in permeability to be real, but it is not necessary to assume a real difference in permeability to account for the effect, for the system is not symmetrical in the two cases, i.e. when sucrose is at the larger end and water at the smaller, and vice versa. From equation (2) it can be seen that the turgor pressure is much lower when the sucrose is surrounding the larger end than when it surrounds the smaller end. Another source of asymmetry arises from the fact that when the smaller end is in sucrose, the velocity of water flow through the area in sucrose is much greater. This leads to a greater concentration of solutes at the internal surface close to the tonoplast and to a greater dilution of the sucrose outside the cell wall and, particularly, *in the wall spaces*. This effect is not nearly so pronounced when sucrose is at the larger end. The overall effect is to reduce the osmotic gradient more when sucrose is at the short end and, with it, the apparent permeability.

We are satisfied that this effect of the increased velocity of water flow is an adequate explanation of the apparent difference in permeability for water moving in and out. This conclusion was reinforced by two further observations. The unequal-areas experiment was repeated with a more dilute sucrose solution (0·1M, which would halve the velocity of water flow) and the ratio of the apparent permeabilities was substantially decreased. Also, using equal areas, P_{os} was constant for osmotic gradients up to 8 atm (cf. Fig. 4, Table 1) but was apparently reduced with greater gradients, i.e. at increasing velocities of flow.

It is concluded, therefore, that there is no difference between the permeabilities for water moving in and out. The apparent difference arises because the osmotic gradients are different from what they are assumed to be in calculating P_{os}.

The osmotic permeability of other plant cells has been measured by plasmolysis and deplasmolysis (e.g. Levitt, Scarth, and Gibbs 1936; Mercer and Clark, unpublished data) and the values found were of the order 1–10 $\mu^3/\mu^2 \,.\, \text{min} \,.\, \text{atm}$. However, Mercer and Clark found that P_{os} of isolated tonoplasts plus vacuoles was up to 10 times that of isolated protoplasts. This suggests that the resistance to water movement is not all in the tonoplast (which is demonstrably differentially permeable) but may be in a plasmalemma, cell wall, or the cytoplasmic layer. With Kamiya and Tazawa, therefore, we stress that the observed permeability constant does not necessarily refer to a particular membrane, in plant cells.

(b) *The Diffusional Permeability*

An attempt was made to measure the rate of exchange of D_2O with ordinary cell water by direct measurement of the change in reduced weight of the cells as $D_2O + H_2O$, of density about 1·05, exchanged with a large volume of H_2O around the cells. This concentration of D_2O (about 50 per cent.) had no apparent effect on the cells as evidenced by normal protoplasmic streaming. However, for the following reasons it was impossible to place reliance on the rates of equilibration so measured:

(i) It is not known whether the external medium is "stirred" or "unstirred".* Vigorous stirring can cause a marked increase in the rate of equilibration.

(ii) If P_d is similar to P_{os}, i.e. of the order of 100 μ/sec, it is clear that the equilibration of a cylinder of diameter 1·0 mm will be rate-limited by the internal diffusion of D_2O up to the cell "membrane". This is apparent when it is considered that a diffusional permeability of 100 μ/sec would allow a half-time for D_2O–H_2O equilibration of 2 sec across the surface of such a cylinder, whereas a cylinder of H_2O of diameter 1 mm equilibrates with a half-time of 6·9 sec (stirred) (Harris 1956, p. 95) or 19·5 sec (unstirred) (Crank 1956, p. 29) assuming a coefficient of self-diffusion of $2 \cdot 3 \times 10^{-5}$ cm^2 sec^{-1} at 19°C (Wang 1954). Even if P_d were of the order of 10 μ/sec it would be difficult to separate the effect of a differentially permeable barrier from that of slow internal diffusion. A comparison of rates of equilibration of cells with those of 2 per cent. agar rods in the same partially stirred condition has revealed very similar rate constants: the half-time of equilibration was about 50 sec. It is therefore impossible to measure the diffusional permeability of these cells in this manner.

These considerations also apply to the experiments made by Wartiowarra (1944) with *Tolypellopsis* and those of Nevis (1958) with squid nerves of diameter *c.* 0·4 mm. Neither of these authors took into account the effect of internal diffusion. However, Paganelli and Solomon (1957) showed that, with red blood cells, where the surface area to volume ratio was very much greater, internal diffusion was not rate-limiting.

(*c*) *Comparison between* $\mathrm{P_{os}}$ *and* $\mathrm{P_d}$

Several workers have interpreted the difference between P_{os} (often termed filtration permeability (P_f) in the literature) and P_d as due to the presence of pores in a membrane controlling water movement. In these schemes water is envisaged as passing by "bulk flow" along the pores under the osmotic pressure difference, whereas during diffusion a labelled water molecule has to move the full length of such a pore by thermal agitation before it appears in the external medium.

If the pore diameter is greater than about 15 Å, it is probably reasonable to associate P_{os} with hydrodynamic flow according to Poiseuille's law or some suitable modification of it (Pappenheimer 1953), and hence assign an approximate pore size from the ratio P_{os}/P_d. Such an analysis is probably valid for water flow through such membranes as frog skin and blood capillary walls and possibly for the cell membrane of the frog ovarian egg.

---

* Hicks (personal communication) has pointed out that a density difference of 0·05 between the D_2O–H_2O mixture appearing at the periphery of the cell and the bulk phase is equivalent to a temperature difference of 110°C between the two, and therefore there is some stirring because of convection.

However, the normal cell membrane is rather impermeable, and the tonoplast very impermeable, to small ions; this indicates that the pores must be quite narrow, in fact roughly equal to the diameter of a water molecule. An osmotic gradient would then result in a moving *file* of water through the pores and Harris (1956, p. 37) and Hodgkin and Keynes (1955) have shown that this situation leads to a lower value for the tracer flux (which gives P_d) than for the real flux. Thus the discrepancy between P_{os} and P_d for water movement across cell membranes which are relatively impermeable to small ions is analogous to the discrepancy between the ion permeabilities of say, squid nerve as measured by membrane conductivity and tracer experiments.

A general quantitative treatment is needed to describe the condition where the pore diameter is greater than the diameter of a water molecule. With plant cells, where the resistance to water movement cannot necessarily be ascribed to a single membrane, the situation is yet more complex.

VI. Acknowledgments

The authors express their thanks to Mr. E. W. Hicks, Division of Food Preservation and Transport, C.S.I.R.O., for fruitful discussions on diffusion; to Dr. W. J. Scott and Dr. J. H. B. Christian, both of the same Division, for helpful criticisms of the manuscript; and to Dr. J. R. Vickery, Chief, Division of Food Preservation and Transport, and Professor R. L. Crocker, Professor of Botany, University of Sydney, in whose laboratories the work was done.

One of us (J.D.) gratefully acknowledges the receipt of a Nuffield–Royal Society Commonwealth Bursary.

VII. References

Crank, J. (1956).—"The Mathematics of Diffusion." (The Clarendon Press: Oxford.)

Harris, E. J. (1956).—"Transport and Accumulation in Biological Systems." (Butterworths Scientific Publications: London.)

Hodgkin, A. L., and Keynes, R. D. (1955).—*J. Physiol.* **128**: 61.

Kamiya, N., and Kuroda, K. (1956).—*Protoplasma* **46**: 423.

Kamiya, N., and Tazawa, M. (1956).—*Protoplasma* **46**: 394.

Levitt, J., Scarth, G. W., and Gibbs, R. D. (1936).—*Protoplasma* **26**: 237.

Løvtrup, S., and Pigón, A. (1951).—*C.R. Lab. Carlsberg* **28**: 1.

MacRobbie, Enid A. C., and Dainty, J. (1958).—*J. Gen. Physiol.* **42**: 335.

Mercer, F. V. (1955).—*Proc. Linn. Soc. N.S.W.* **30**: 6.

Nevis, A. H. (1958).—*J. Gen. Physiol.* **41**: 927.

Osterhout, W. J. V. (1949*a*).—*J. Gen. Physiol.* **32**: 553.

Osterhout, W. J. V. (1949*b*).—*J. Gen. Physiol.* **32**: 559.

Paganelli, C. V., and Solomon, A. K. (1957).—*J. Gen. Physiol.* **41**: 259.

Palva, P. (1939).—*Protoplasma* **32**: 265.

Pappenheimer, J. R. (1953).—*Physiol. Rev.* **33**: 387.

Prescott, D. M., and Zeuthen, E. (1953).—*Acta Physiol. Scand.* **28**: 77.

Wang, J. H. (1954).—*J. Amer. Chem. Soc.* **76**: 4763.

Wartiowaara, V. (1944).—*Acta Bot. Fenn.* **34**: 1.

PHYSIOLOGY OF CEREAL GRAIN

II. STARCH GRANULE FORMATION IN THE DEVELOPING BARLEY KERNEL*

By L. H. MAY† and M. S. BUTTROSE†

[*Manuscript received December* 11, 1958]

Summary

Types, numbers, volumes, and weights of starch granules in the barley endosperm were measured at different times from anthesis to maturity. The formation of two types of granule was confirmed: the first (type A) was initiated until 15 days after anthesis; the second (type B) between 18 and 30 days. At maturity there were approximately 10 times as many type B granules as type A, although the latter made up 90 per cent. of the total granule volume. There was a linear relationship between starch granule and endosperm volume throughout kernel development, while the rate of increase in volume per unit granule volume was the same, irrespective of granule size, at any one time. Starch weight increased as endosperm dry weight increased although the precise form of this relationship is in doubt. The interrelationships between starch granule weight and volume, and also endosperm dry weight and volume, suggest that both starch granules and endosperm increase in density during development.

Two enzyme activities (the one producing maltose from starch, the other inorganic phosphate from glucose 1-phosphate) were measured relative to each other at different times from anthesis to maturity. A regular change was found in this relationship, which was independent of the individual levels of activity.

The significance of these results is discussed with respect to the mechanisms regulating different aspects of starch granule formation.

I. INTRODUCTION

Starch is deposited by plants as organized structures called granules, the form of which is often characteristic of the species. Modern concepts of granule formation are embodied in reviews by Alsberg (1938), Frey-Wyssling (1953), and Badenhuizen (1958).

It is generally agreed that the layers, or shells, which characterize granule structure arise as a result of rhythmic forces. There is no general agreement, however, that they are related to the periodicity of photosynthesis as originally proposed by Meyer (1895), or indeed that they are related to any other diurnal rhythm. If this were true it could be concluded that a granule grows over the number of days corresponding to the number of shells. Detailed evidence to test this hypothesis has proved difficult to obtain. Sandstedt (1946) has reported that starch granules in the wheat pericarp increase in both number and size up to 7 days after flowering, and that thereafter they decline. In the endosperm granules appear first at 4 days.

---

* Some of the results recorded in this paper were included in the thesis presented by one of us (M.S.B.) for the degree of M.Sc. in the University of Adelaide.

† Waite Agricultural Research Institute, University of Adelaide.

They are small, transparent, and bean-shaped and grow rapidly, some attaining diameters of 35 μ at maturity. At 14 days a different type of granule appears. These are small and spherical and grow no larger than 10 μ in diameter. Harlan (1920) has reported a uniform development of granules in barley up to 15 days followed by a continued development of only some larger granules. Finally, in maize, a general shift in relative frequency distribution of granules into higher size groups has been observed throughout development of the endosperm (Wolf *et al.* 1948). However, these observations lack sufficient detail to test the hypothesis. Recent electron-micrographs of Muhlethaler (1955) show about 20–25 shells in mature maize granules, a number which seems consistent with the hypothesis that each shell represents one day's increment, but comprehensive information on the number of shells is scarce.

The experiments reported here were carried out to throw some further light on the general pattern of starch granule development. To this end the rate of initiation of starch granules, the rate of granule growth, and some changes in enzymic activity were followed in the barley endosperm.

II. METHODS

(*a*) *Culture Techniques and Sampling*

A two-row barley (*Hordeum distichum* var. Prior), which is a local selection widely grown in South Australia, was used in this work. Seeds (36 per pot) were planted in sand, and, after thinning (to 30 per pot), culture conditions were as described by Buttrose and May (1959). Only the main shoot was allowed to develop, tillers being cut off as they appeared.

Ears were tagged on the day that anthesis occurred. Heads (48) which underwent anthesis on the same day were assigned at random to each of 12 harvest occasions (four per occasion). Harvests were at 3-day intervals to 30 days, then at 36 and 42 days after anthesis. For starch granule counting three kernels from each of the four heads were arbitrarily selected as the third and fifth lowest on one side of the head, and the fourth lowest on the other. For dry weight and volume determinations five kernels from each of the four heads were used: the fourth and sixth lowest on the one side and the third, fifth, and sixth on the other.

Enzyme studies were conducted on a different set of plants (six per pot) which were allowed to develop tillers. Ears from all shoots were used. Culture conditions were as above. Heads which underwent anthesis on the same day were harvested on six occasions: at 10, 17, 24, 30, 34, and 58 days after anthesis.

(*b*) *Granule Counting*

Kernels (the three from each head were combined and treated as one sample) were freed of glumes and the endosperms separated and ground in a mortar and pestle with 1 ml iodine solution (0·2 per cent. iodine in 2 per cent. potassium iodide) for 5 min. The suspension was transferred, with distilled water, to a graduated tube and made to a suitable volume (5 or 10 ml) with iodine solution (1 ml) and water.

After vigorous shaking a portion was drawn into a wide-bore pipette (1 ml) the tip of which was held mid-way down the suspension. Then the pipette was held horizontally, to prevent differential sedimentation relative to the tip, and a suitably small drop was transferred to a haemocytometer counting slide. The coverslip was immediately placed in position. It was found necessary to standardize all of these steps and to perform them as rapidly as possible; furthermore, the site of the original drop was always checked to determine whether any excess sedimentation had occurred there. Careful observation over several hours failed to reveal any granule swelling in water, and swelling during the initial dispersion is considered unlikely since substitution of water with sucrose solution (5 per cent.) did not affect granule size. Measurements were made at a magnification of 400 times on five evenly spaced fields of the haemocytometer grid. The dilutions gave at least 30 granules per field and the diameter of every granule larger than $6 \cdot 7\ \mu$ was measured with a micrometer eyepiece. Where the granule was not circular the mean of major and minor diameters was recorded. Granules less than $6 \cdot 7\ \mu$ were counted and placed within the two ranges $0–3 \cdot 3\ \mu$ and $3 \cdot 3–6 \cdot 7\ \mu$ by visual assessment. After 18 days from anthesis the numbers within the latter two size groups were so large that a second dilution of the original suspension was used for their estimation.

The above procedure was repeated using a further portion from the original suspension. Results obtained from four heads were then combined and expressed as numbers of granules per endosperm per size group. Each size group covered $3 \cdot 3\ \mu$ diameter. Starch granules in the pericarp tissues were examined in the same way.

(c) *Volume and Dry Weight*

Kernels were freed of glumes and the volume determined by displacement of water in a graduated tube, the displacement being measured with a vertically travelling microscope. The endosperm was then dissected and its volume measured. Estimates made at 1-min intervals suggested that endosperm volume was not altered during the period of measurement.

Dry weights were obtained by taking to constant weight in a vacuum desiccator. This procedure gave similar results to oven drying and was employed since it subsequently allowed easier extraction of starch.

(d) *Chemical Analysis of Starch*

The vacuum-dried sample was ground lightly in a mortar and pestle, transferred quantitatively to an extraction thimble, and extracted 10 times with ethanol (80 per cent.) in a Soxhlet extraction apparatus. The residue was extracted six times with water (below 30°C), dried in a vacuum desiccator, removed from the thimble, and weighed. Ethanol and water extracts were found to be free from starch. The residue was ground thoroughly in a mortar and pestle for 10 min and subsamples (20–50 mg) were extracted in centrifuge tubes with chloral hydrate (3 ml, 33 per cent.) at 80°C for 1 hr with frequent stirring. The tubes were centrifuged and the supernatant decanted. There were two further extractions with chloral hydrate (1 ml), the contents being stirred at 80°C for 15 min. The combined extracts were made up to 5 ml with water, well mixed, and a portion (1 ml) injected with shaking into acetone (5 ml) in a centrifuge tube. The starch precipitate was collected by centrifugation and washed twice with acetone (2 ml) and twice with ether (2 ml). When dry, the starch was hydrolysed with normal sulphuric acid (2 ml) at 100°C for 3 hr. Excess acid was then neutralized with barium carbonate. The reducing power was determined by Somogyi's (1952) modification of the method of Nelson (1944). Each sample was estimated in duplicate and appropriate blanks and standards included.

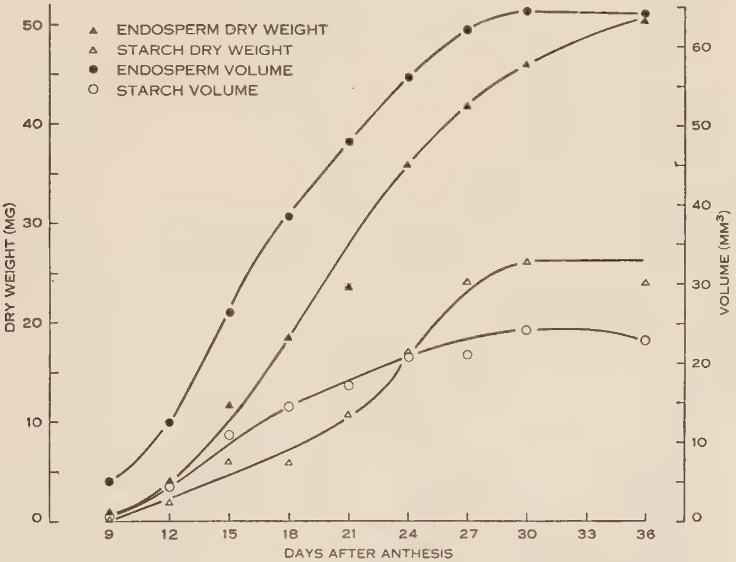


Fig. 1.—Weight and volume changes of the endosperm and starch granules during development of the barley kernel.

(e) *Enzyme Assays*

(i) *Preparation of Extracts*.—Kernels (50) were taken from the harvested heads, and glumes, awns, and embryos were removed. The remaining endosperm was ground with acid-washed sand and sodium citrate buffer (10 ml, pH 6·7), transferred to a centrifuge tube, and stored at 3°C overnight. After centrifuging at 4000 *g* for 1 hr portions were taken for enzyme assays.

(ii) *Measurement of Enzyme Activity*.—Digests contained a mixture of glucose 1-phosphate (0·3 ml, 0·038M), starch (4 ml, 1 per cent.), sodium citrate buffer (2 ml, pH 6·7), endosperm extract (0·2 ml), and water to make up to 10 ml. On each occasion appropriate controls were included.

For estimations of reducing sugars immediately the endosperm extract was added and the digest mixed, a portion was delivered into mercuric chloride (0·5 ml, 0·0004M) to stop the reaction. This procedure was repeated after 15, 30, and 60 min and after 24 hr. Maltose was estimated from its reducing power as previously described. Separation by chromatographic techniques of the sugars formed showed that maltose was the only sugar produced in appreciable amounts. This result together with the fact that conversion of starch to maltose rarely exceeded 60 per cent. at 24 hr, and changed little thereafter, supports the view that β-amylase is the major enzyme degrading starch under these conditions. For estimations of inorganic phosphate portions were withdrawn from the original digests at 30 min, 24, and 48 hr, and estimated according to Fiske and Subarrow (1925).

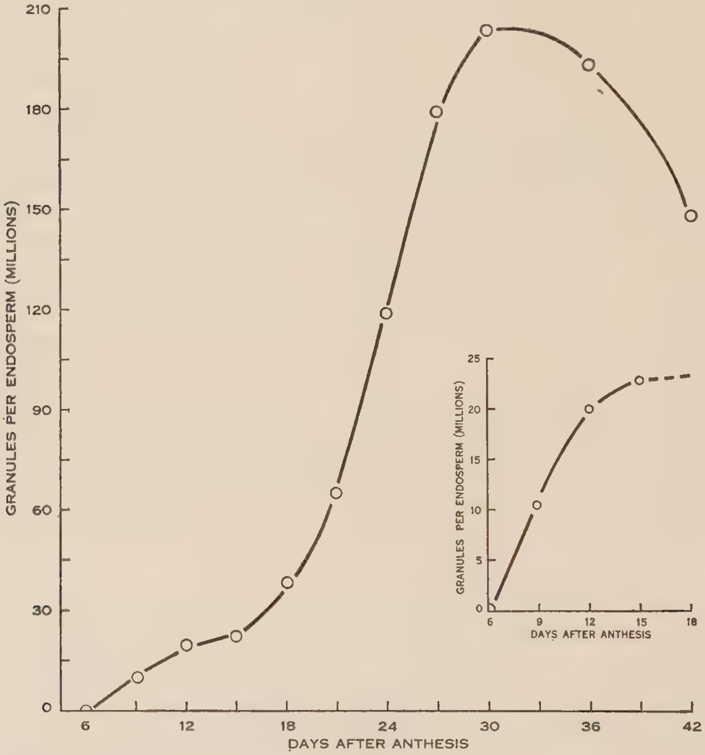


Fig. 2.—Increase in total number of starch granules during development of the barley endosperm. *Inset.*—The initial part of the curve enlarged and continued to estimate the maximum number of type A granules produced (see text).

All digests were maintained under toluene liquid at 30°C.

(iii) *Comparison of Enzyme Activities.*—These are expressed in the following units: firstly, the percentage of phosphate released from glucose 1-phosphate after 24 hr; secondly, the percentage of starch converted to maltose in 15 min. Preliminary experiments using ammonium molybdate (1 ml, 0·018M) in some digests suggest that phosphatase contributed significantly to the release of phosphate but further analysis of this effect is not attempted here.

III. Results

(a) *Endosperm Development*

(i) *Endosperm Volume and Dry Weight.*—From 9 days after flowering until 30 days the volume per endosperm increased; thereafter it fell slightly as the kernel dried (Fig. 1). The dry weight per endosperm increased more slowly until at 36 days after flowering it reached a maximum.

(ii) *Numbers and Types of Starch Granules.*—The numbers of granules present in the endosperm during development are shown in Figure 2. The change in slope of the curve at 15 days coincides with an observed change in the type of starch granule produced. Granules initiated up to 15 days from anthesis (type A) were at first lenticular-shaped, irregular in outline, and quite transparent, whereas those initiated between 18 and 30 days (type B) were spherical, regular in outline, and much denser. Continuation of the initial curve from 15 days onwards (see *inset*, Fig. 2) indicates a maximum of $23 \cdot 5 \times 10^6$ granules per endosperm which approximates the number of granules at maturity that had a diameter greater than $6 \cdot 7\ \mu$ ($20 \cdot 6 \pm 1 \cdot 0 \times 10^6$). It seems reasonable to assume, therefore, that the largest granules at maturity were type A. Between 18 and 30 days large numbers of particles, similar to type B granules although lacking in starch, appeared. However, neither these particles nor type B granules were observed before 15 days.

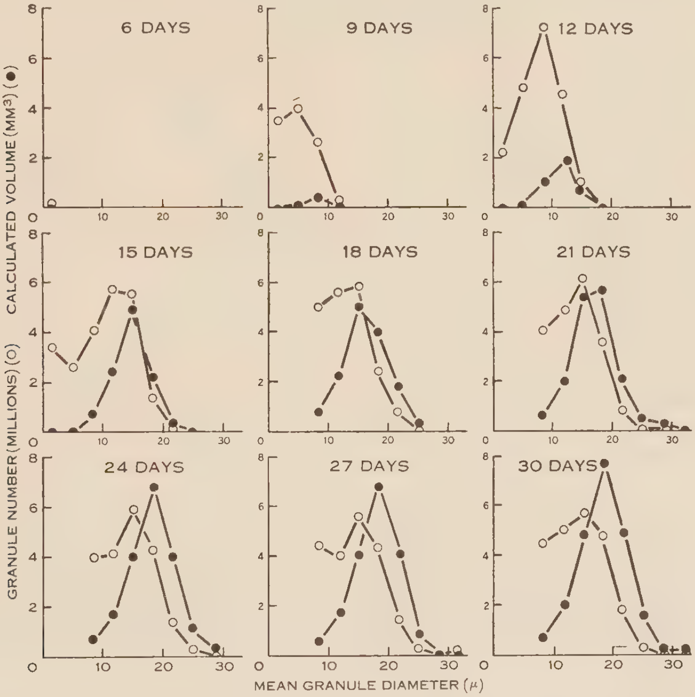


Fig. 3.—Frequency and volume distributions of starch granules (type A) during development of the barley endosperm. The size groups increase in diameter by $3 \cdot 3\ \mu$.

After 30 days there is an apparent reduction in granule number. It is tentatively suggested that this is due to the incomplete dispersion of aggregates of small granules since after 30 days the preparation of a satisfactory dispersion for counting became difficult.

(iii) *Starch Granule Volume and Weight.*—The shape of type A granules, when measured as the ratio of the major to minor axis, does not change during development. Calculations based on models of granules indicate that $\frac{2}{3}\pi r^3$ approximates to their volume, where r is the major radius. The mid dimensions of each granule size group was used as a basis for calculating the total volume of granules in that group. Frequency and volume distributions of type A granules at different stages during development are shown in Figure 3. Summation of the individual group volumes gave an estimate of the total volume of type A granules at 3-day intervals throughout kernel development, and these values, added to the volumes of granules less than $6 \cdot 7\ \mu$ in diameter, have been plotted in Figure 1. Trends in starch dry weight throughout development have also been included in this figure. At all stages type A granules contributed more than 90 per cent. of the total volume (despite the fact that type B granules represent 88 per cent. of the total numbers at 36 days), and it is concluded that growth of type A granules continued through to maturity. This conclusion is supported by the steady shift in distribution from smaller to larger size groups (Fig. 3). Because of the much smaller size of type B granules, present techniques do not allow a decision as to whether they also developed continuously after initiation. Nevertheless, the results for type A granules are not

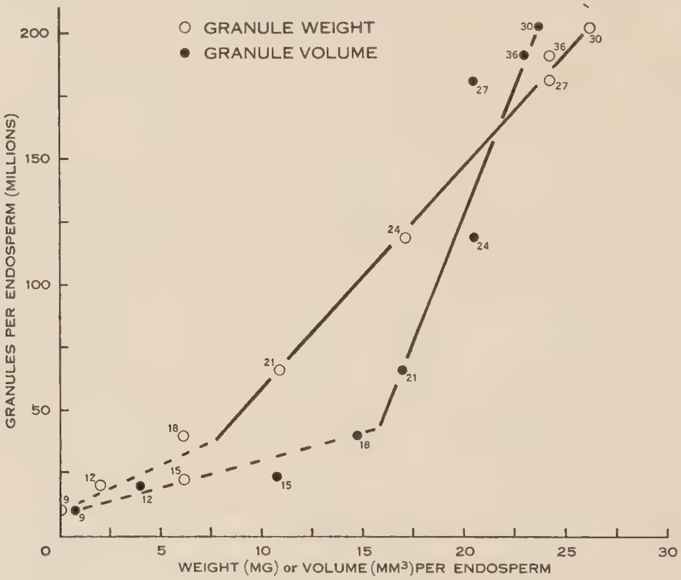


Fig. 4.—Interrelationships between: number of granules and starch granule volume, number of granules and weight of starch. The figures refer to the time from anthesis (in days) when the estimates were made.

consistent with the report by Bice, MacMasters, and Hilbert (1945) that granule size in wheat did not increase after 16 days.

The total number of granules is clearly related to both starch volume and starch weight after 15 days (Fig. 4). Before 15 days, there are too few estimates to establish whether or not a significant correlation exists.

(iv) *Interrelationships between Weights and Volumes.*—Interrelationships between granule weight and endosperm dry weight and between total granule volume and endosperm volume are illustrated in Figures 5(*a*) and 5(*b*) respectively. The volumes are linearly related over the whole period of development and a significant linear regression line can also be fitted to the corresponding starch and endosperm weights, but this latter relationship is further examined below (see Section IV).

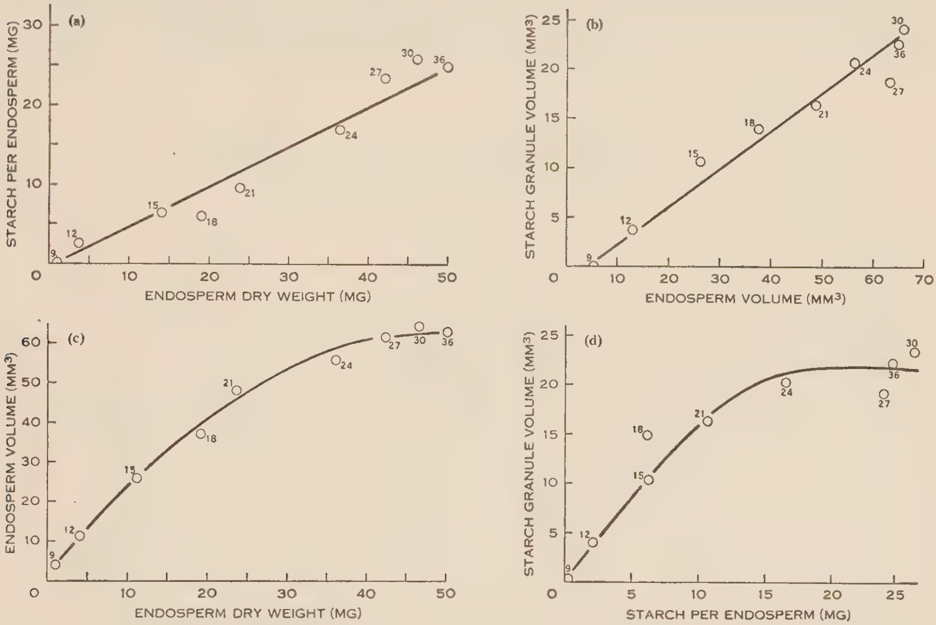


Fig. 5.—Some interrelationships during development of the barley endosperm. The figures refer to the time from anthesis (in days) when the estimates were made. (*a*) Weight of starch per endosperm and endosperm weight. (*b*) Total starch granule volume and endosperm volume. (*c*) Volume and weight of endosperm. (*d*) Volume and weight of starch granules in the endosperm.

Interrelationships between the volume and the weight of endosperm and of starch granules are illustrated in Figures 5(*c*) and 5(*d*) respectively. In both instances volume increases rapidly with weight at first, but more slowly later. In other words both starch and endosperm density increases in the later stages of development, presumably through the displacement of water with denser materials. However, their densities are not the same. A maximum value for starch weight of 27·5 mg (Fig. 1) is 55 per cent. of the total dry weight; calculated starch granule volume comprises only 38 per cent. of the total volume. This result suggests that starch granules are denser than non-granular materials in the endosperm and has support from the findings that granules sink in sucrose solutions of density 1·4 whereas the density of whole fresh endosperm is less than 1·25.

(*b*) *Pericarp Development*

Details of starch granule formation in, and general growth of, pericarp tissues are presented in Table 1. Numbers and size of granules increase mainly before 6 days from anthesis, and decrease rapidly after 12 days. Increase in dry weight and volume occurs up to 12–15 days; thereafter there is little change.

The amount of endosperm starch at 9 days exceeds that of the pericarp and the rapid increase in the former from 9–12 days is not compensated by any decrease in the latter. Furthermore, the reduction in volume of pericarp starch from 12–15 days represents only 1 per cent. of the increase in endosperm starch volume during this time. It seems unlikely, therefore, that pericarp tissue represents a significant source of substrate for endosperm development, although whether the development of the endosperm inhibits further pericarp development either directly or indirectly remains to be settled.

TABLE 1

NUMBERS OF STARCH GRANULES IN PERICARP TISSUES AND THE DRY WEIGHT AND VOLUME OF THESE TISSUES DURING DEVELOPMENT

— = not assessed

Time from Anthesis (days)	Starch Granule Number (millions)			Dry Weight (mg)	Volume (mm³)
	Diameter 0–3·3 μ	Diameter 3·3–6·7 μ	Total		
3	3·8	1·6	5·4	0·8	4·2
6	9·2	2·0	11·2	2·4	10·5
9	9·2	1·9	11·1	3·8	13·2
12	8·0	2·1	10·1	3·7	20·2
15	2·4	0·4	2·8	2·4	14·9
18	—	—	—	3·4	17·0
21	—	—	—	4·7	23·9

(*c*) *Enzyme Changes in Endosperm and Pericarp*

Two enzymic activities were measured: maltose production from starch and inorganic phosphate production from glucose 1-phosphate. Both activities (expressed in units per endosperm—see Section II(*e*)(iii)), rose between 10–17 days after anthesis, thereafter the former activity continued to rise slowly while the latter declined (Fig. 6(*a*)). However, the ratio, inorganic phosphate formed to maltose formed, changed in a regular manner throughout (Fig. 6(*b*)).

IV. Discussion

The formation of two types of starch granule in cereal endosperm has been confirmed by this work. Badenhuizen (1958) has suggested that the first granules formed (type A) originate in normal plastids, whereas the second (type B) arise in mitochondria. In any event it seems that some major change takes place in cell organization at 15 days after anthesis.

Although type A granules were all initiated within a relatively short time (about 6 days) they ranged widely in size at maturity, e.g. granules of 27 μ diameter cannot have begun development much earlier than granules of 18 μ, yet the former occupied three times the volume of the latter. There are several possible explanations. Firstly, some granules may have a greater inherent capacity for growth than others. Secondly, the location of granules in the endosperm may influence the availability of substrate. Thirdly, the relative growth rate of granules may be critically determined initially by their time of initiation within the 6-day period, and may be independent subsequently of their size. The present work lends no support to either of the first two proposals. To examine the third proposal the rates of increase of volume per unit volume were calculated for total starch granules, and also for granules formed 6–9 and 9–12 days after anthesis (Fig. 7(*a*)). The calculations for the two groups of early-formed granules were based on the following assumptions. The $10 \cdot 3 \times 10^6$ granules present at 9 days were assumed to remain the largest granules at all subsequent stages, their total volume was obtained for each harvest (see Fig. 3), and the volume of the average granule calcu-

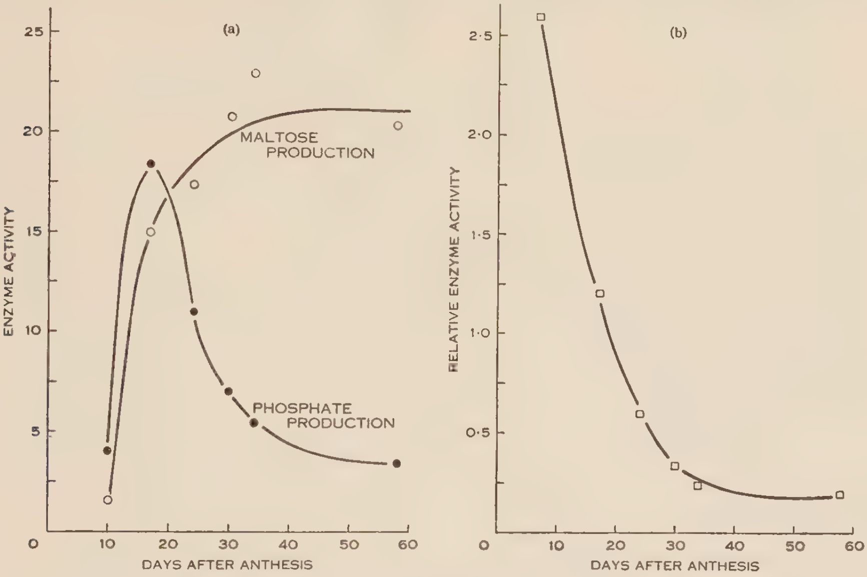


Fig. 6.—Estimates of activity of endosperm in catalysing two reactions (maltose production from starch; phosphate production from glucose 1-phosphate) at progressive stages during the development of the kernel. (*a*) Individual activities per endosperm (for units see text). (*b*) Ratio of inorganic phosphate formed to maltose formed.

lated. It was further assumed that the $9 \cdot 8 \times 10^6$ granules appearing between 9 and 12 days remained, during subsequent development, immediately below the previous group in size. Again, the average volume at all stages was calculated for these granules. If these assumptions are accepted then it can be seen that at any one time all granules, irrespective of their size, increase in volume per unit volume at the same rate and furthermore that this rate decreases with time. It follows that granules initiated at 6 days have an advantage over granules initiated at 9 days which is never offset; on the contrary the earlier-formed granules increase in volume (in absolute terms) progressively faster than later-formed ones.

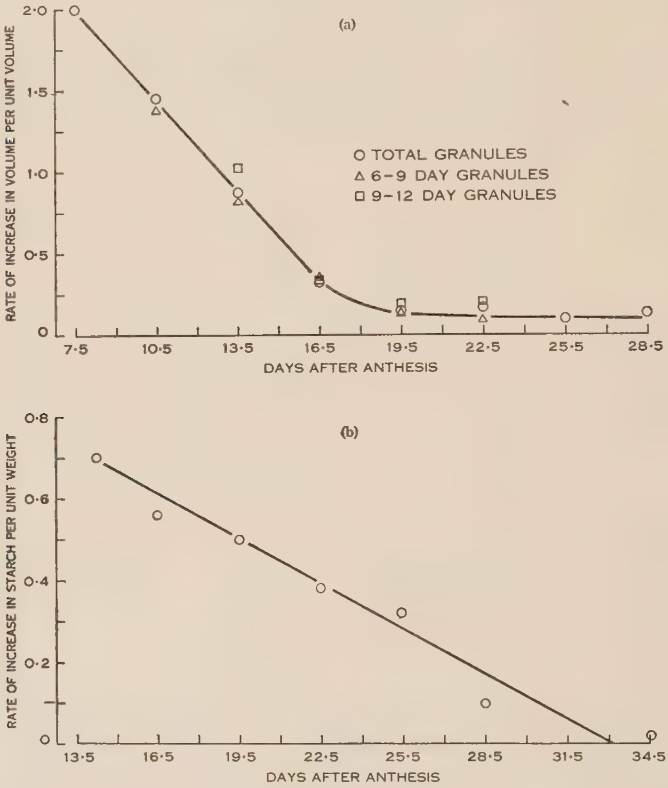


Fig. 7.—(*a*) Relative growth rate (volume) of total starch granules, those initiated 6–9 days from anthesis, and those initiated 9–12 days from anthesis during their development within the barley endosperm. (*b*) Relative growth rate (weight) of total starch granules during development within the barley endosperm.

The rate of increase of starch weight per unit weight decreases with time as does the comparable volume factor, but in this case the rate declines rather more uniformly (Fig. 7(*b*)). In absolute terms starch weight increases as endosperm dry weight increases. When the individual estimates are plotted a significant linear regression line can be fitted (Fig. 5(*a*)). When values are taken from the two smoothed curves of starch weight and endosperm weight (Fig. 1) there appears to be a two-stage relationship the form of which suggests that the proportion of total endosperm dry weight being channelled into starch before 21 days is less than that after this time.

It has been noted (Figs. 5(*c*) and 5(*d*)) that starch granules, and the endosperm, increase in density with time. This conclusion is in agreement with observations of other researchers (Bice, MacMasters, and Hilbert 1945; Sandstedt 1946), who concluded that young granules are less dense and more hydrated than older ones. In addition Sandstedt (1955) and Nikuni and Whistler (1957) have shown that the central portions of granules are more susceptible to enzyme digestion than the periphery and hence, it is claimed, the centre is the more loosely packed. Furthermore, Sandstedt (1946) noted a centrally placed lenticular area in lintnerized wheat granules. This observation was confirmed in the present work with barley, the area corresponding in size and shape with a young granule about 2 days old. On the assumption that growth of granules is always by apposition, the growth of the two granules previously discussed (one initiated 6–9 days, the other 9–12 days) may be represented diagrammatically as in Figure 8 where each day's increment is represented by a ring. Daily increments were derived from the smoothed curves of volume increase obtained for 3-day periods throughout development (see discussion on Fig. 7). If there is a diurnal rhythm of starch deposition, which is reflected in the shells in granules, then layers seen in mature granules of these diameters should correspond with the rings illustrated here. An electron-micrograph of a maize granule published by Nikuni and Whistler (1957) shows a closer spacing of rings towards the periphery, but there is no indication of their relative numbers.

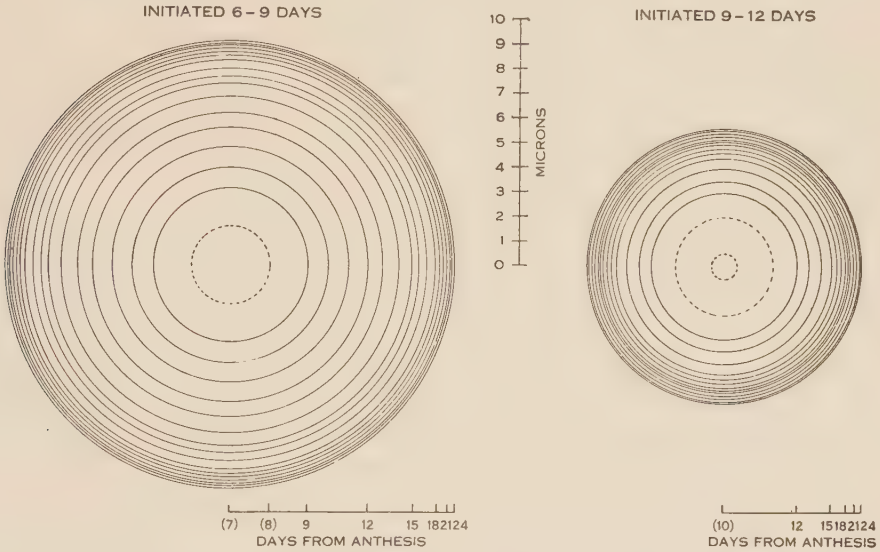


Fig. 8.—Diagrammatic representation of the calculated daily growth in cross section of an average starch granule initiated either 6–9 or 9–12 days from anthesis.

Whereas starch granule development in terms of volume, number, type, and weight is now more clearly defined, the mechanisms regulating these changes are still largely matters for speculation. There seems no *a priori* reason for supposing that the same factor regulates all aspects of starch granule development and, therefore, they will be considered separately.

The main features of volume development that emerge from this work are firstly, that rate of volume increase per unit volume is the same at any one time irrespective of granule size, and secondly, that this rate decreases with time. The significant correlation between endosperm volume and starch granule volume makes it tempting to suggest that the former controls the latter. This suggestion receives some support from the proposal by Evans (1941) that the close packing of maize endosperm granules is responsible for their polygonal shape at maturity. Again, indentations on mature wheat granules are attributed to pressure from small granules (Bice, MacMasters, and Hilbert 1945; Sandstedt 1946). Control by the endosperm could account for the rate of volume increase per unit volume being independent of the size of granules, and furthermore the reasons for the decreasing relative rate of volume increase with time would be associated with those factors limiting endosperm development as a whole and not the starch granules themselves.

The relationships between numbers of starch granules and either starch volume or starch weight are also of interest. On the one hand the number of type A granules initiated (possibly in plastids) before 15 days may be under hereditary control. On the other hand numbers of type B granules are directly related to the volume and weight of starch (Fig. 4). Again spacial control by the endosperm offers a plausible explanation since maximum utilization of space, once different granule sizes have become established, requires an increasing formation of smaller and smaller granules.

The rate of weight increase per unit weight decreases in a linear fashion with time. The following have been assessed for their role as controlling factors: substrate, space, receptor units, enzyme activity. Previous work (Buttrose and May 1959) and the results presented here do not support any of the first three factors. However, strong support for the role of enzyme activity has come from the findings of Turner and Turner (1957) who established a linear relationship between phosphorylase activity and rate of starch synthesis during most of the time pea seeds are developing. Nevertheless, as they point out, the role of other enzymes cannot be ignored; the synthesis of amylopectin requires the participation of at least one further enzyme, and others may be concerned with the organization into granules (Whelan 1958). There is a growing body of evidence that enzymes change in their activities relative to each other during the development of plant organs, and increasing weight is being given to the view that such changes may be as important in regulating metabolism as changes in absolute levels of activity. The demonstrations by Porter (1950, 1953) that β-amylase is an inhibitor of starch phosphorylase and that phosphatase may be depressed by high phosphorylase levels make it clear that the estimates obtained in this work cannot be regarded as indicating the absolute levels of any enzymic activity. However, this was not the aim. What the present work offers is one further example of a change during development in the ability of an extract to catalyse one reaction relative to its ability to catalyse another, whatever the enzymes responsible.

All aspects of granule growth (volume, number, type, and weight) are probably more closely interrelated than has been emphasized in this discussion. However, it is manifest that much further investigation is required before the factors regulating the development of one of the simplest biological macromolecules can be properly integrated.

V. Acknowledgment

The authors wish to thank Mrs. M. M. Semmens for technical assistance in conducting the enzyme assays.

VI. References

Alsberg, C. L. (1938).—*Plant Physiol.* **13**: 295.

Badenhuizen, N. P. (1958).—"Encyclopedia of Plant Physiology." Vol. 6. p. 137. (Ed. W. Ruhland.) (Springer-Verlag: Berlin.)

Bice, C. W., MacMasters, M. M., and Hilbert, G. E. (1945).—*Cereal Chem.* **22**: 463.

Buttrose, M. S., and May, L. H. (1959).—*Aust. J. Biol. Sci.* **12**(1): 40.

Evans, J. W. (1941).—*Cereal Chem.* **18**: 462.

Fiske, C. H., and Subarrow, Y. (1925).—*J. Biol. Chem.* **66**: 375.

Frey-Wyssling, A. (1953).—"Submicroscopic Morphology of Protoplasm." (Elsevier Publ Co.: Amsterdam.)

Harlan, H. V. (1920).—*J. Agric. Res.* **19**: 393.

Meyer, A. (1895).—"Untersuchungen uber die Starkekorner." (Gustav Fischer: Jena.)

Muhlethaler, K. (1955).—*Z. wiss. Mikr.* **62**: 394.

Nelson, N. (1944).—*J. Biol. Chem.* **153**: 375.

Nikuni, Z., and Whistler, R. L. (1957).—*J. Biochem., Tokyo* **44**: 227.

Porter, H. K. (1950).—*Biochem. J.* **47**: 476.

Porter, H. K. (1953).—*J. Exp. Bot.* **4**: 44.

Sandstedt, R. M. (1946).—*Cereal Chem.* **23**: 337.

Sandstedt, R. M. (1955).—*Brew. Dig.* **30**: 49.

Somogyi, M. (1952).—*J. Biol. Chem.* **195**: 19.

Turner, D. H., and Turner, J. F. (1957).—*Aust. J. Biol. Sci.* **10**: 302.

Whelan, W. J. (1958).—"Encyclopedia of Plant Physiology." Vol. 6. p. 154. (Ed. W. Ruhland.) (Springer-Verlag: Berlin.)

Wolf, M. J., MacMasters, M. M., Hubbard, J. E., and Rist, C. E. (1948).—*Cereal Chem.* **25**: 312.

THE EFFECT OF CHLORIDE ON THE FREE AMINO ACIDS OF CABBAGE AND CAULIFLOWER PLANTS

By J. R. FRENEY,* C. C. DELWICHE,† and C. M. JOHNSON‡

[*Manuscript received October* 16, 1958]

Summary

Data are presented on the amino acid composition of two varieties of *Brassica oleracea* as influenced by supply of chloride ion to the plant.

The amino acid composition of the two varieties was markedly affected by chloride supply. Amino acid concentrations in plant tops were generally greater in the chloride-deficient plants. Arginine, proline, methionine sulphoxide, glutamine, asparagine, and pipecolic acid accumulated in large amounts in chloride-deficient plants.

Although chloride deficiency has a profound effect on amino acid concentrations in the plant, this effect is not direct evidence for participation of chloride ion in any specific amino acid interconversion.

I. INTRODUCTION

It has been shown that chlorine is essential for the growth of higher plants (Broyer *et al.* 1954; Ulrich and Ohki 1956; Johnson *et al.* 1957; Ozanne, Woolley, and Broyer 1957). The role of chlorine in plant metabolism is not yet known, and the only work relating chloride ion to a specific biochemical reaction in plants is that of Warburg and Lüttgens (1946) suggesting an effect of the ion on the photosynthetic reaction. The work reported here was undertaken to ascertain the possible effect of chlorine on the composition and concentration of the free amino acid fraction in plants.

II. EXPERIMENTAL

(*a*) *Cultural Methods*

Cabbage (*Brassica oleracea* var. *capitata*) and cauliflower (*Brassica oleracea* var. *botrytis*) were cultured in nutrient solutions, with and without added chloride, by the method described by Johnson *et al.* (1957).

Harvests of normal and deficient plants were made when the plants were approximately 7 weeks old. At this time cabbage plants from nutrient solutions without supplemental chloride exhibited typical chloride deficiency symptoms as described previously (Johnson *et al.* 1957), with much restricted growth, cupping of blades, and severe wilting of tips and margins of blades when the plants were exposed to full sunlight.

---

*Division of Plant Industry, C.S.I.R.O., Regional Pastoral Laboratory, Armidale, N.S.W.

†Kearney Foundation of Soil Science, University of California, Berkeley, California.

‡Department of Soils and Plant Nutrition, University of California, Berkeley, California.

Symptoms of chloride deficiency in cauliflower have not previously been described and were different in some respects from those observed in cabbage. In addition to the cupping and wilting characteristic of chloride deficiency in cabbage, the blades of cauliflower were chlorotic. Older blades developed irregular patterns of necrotic tissue, never amounting, however, to more than 25 per cent. of the total area.

Unfortunately, chloride concentrations in the tissues were not determined. However, development of characteristic chloride deficiency symptoms in plants cultured in nutrient solutions without chloride supplements and the absence of these symptoms in plants from solutions with chloride supplements as well as large yield differences with treatment provided ample evidence that changes in amino acid composition of the tissues were reflections of chloride nutritional status.

(*b*) *Analytical Procedure*

Plant tops were extracted immediately upon harvest with a quantity of boiling 95 per cent. ethanol, such that the final concentration of alcohol in the extract was 70 per cent. by volume. Approximately 50 g fresh weight of plant material was taken for each extraction.

Chromatograms were prepared directly from the alcoholic extracts. Preliminary trials showed that purification of the alcoholic extracts by ion-exchange treatment was unnecessary.

Qualitative analyses were carried out on 57 by 46 cm sheets of Whatman No. 1 paper using two-dimensional paper chromatography. The descending method of solvent flow, using a phenol–water mixture (80 : 20 w/v) was used in the first direction, followed by the *n*-butanol–acetic acid–water mixture (4 : 1 : 1 v/v) of Reed (1950) in the second direction. The identity of the various amino acids and amines was established by chromatographing the alcoholic extract along with additions of known amino acids. The results were confirmed by repeating this procedure with two other pairs of solvents. The first pair was *n*-butanol–acetic acid–water (4 : 1 : 5 v/v) in the presence of an atmosphere in equilibrium with a solution of 33 per cent. diethylamine in water; and *tert.*-butanol–water (7 : 3 v/v) in the presence of an atmosphere in equilibrium with a solution of 1 per cent. diethylamine in water (Smith and Tuller 1955). The second pair was pyridine–acetic acid–water (50 : 35 : 15 v/v) (Decker and Riffart 1950) and 95 per cent. ethanol–ammonia (95 : 5 v/v).

After the papers had dried in air under forced draught the spots were developed by dipping in a 0·5 per cent. solution of ninhydrin in acetone (Toennies and Kolb 1951). The papers were then left overnight at room temperature for colour development.

Proline was detected by the isatin reagent of Archer, Fromageot and Jutisz (1950), and the Sakaguchi (1950) reagent was used to confirm the presence of arginine.

Quantitative analyses were carried out by a method similar to that described by Wellington (1952). However, since humidity was not controlled, standards were run with each group of unknowns to aid in obtaining reliable results. Phenol and n-butanol–acetic acid were used as described for the qualitative analyses above. The papers were dried in forced draught at 23°C and then dipped in a 2 per cent. acetone solution of ninhydrin containing 0·1 per cent. pyridine. The colour was developed at 23°C for 24 hr, the spots were cut out, and their colour eluted by shaking with 5 ml of 50 per cent. aqueous ethanol for 10 min. The paper was separated from the solutions by centrifugation. The optical density of the solutions was read at 570 mμ.

Proline was determined separately by the visual comparison technique described by Berry and Cain (1949) after development of colour with isatin (Archer, Fromageot, and Jutisz 1950).

Aliquots of extracts taken for chromatography were adjusted so that the amount of amino acid determined fell within the range of the standards, i.e. between 1 and 15 μg amino acid. In most cases the aliquots taken represented between 16–60 mg fresh plant material. However, for the estimation of methionine sulphoxide, arginine, and proline in chloride-sufficient plant material, it was necessary to use up to the equivalent of 160 mg plant material to obtain a detectable amount of amino acid. Even with this amount of plant material the quantity of these amino acids sometimes fell below the lowest standard. Larger amounts could not be taken for the accurate determination of methionine sulphoxide or arginine in these cases because of the overlapping of spots under these conditions.

Over the range 1–15 μg covered by the standards, the error of a determination for 90 per cent. of the standard samples was less than the figure given in parenthesis: leucine, valine (0·1 μg); α-alanine, glycine (0·2 μg); serine, threonine, γ-aminobutyric acid, methionine sulphoxide (0·3 μg); aspartic acid (0·4 μg); glutamic acid, proline (0·5 μg); pipecolic acid (0·6 μg); glutamine, ethanolamine (0·7 μg); asparagine (0·8 μg); arginine (0·9 μg).

(c) *Statistical Analysis*

In an attempt to equalize variances within treatments for statistical analysis, a square root transformation was made on the primary data obtained from three replicate experiments. Analysis of variance was then carried out on the transformed data.

III. Results and Discussion

As examples of the variation obtained within and between experiments, the replicate values of the asparagine and α-alanine concentrations in cauliflower plants are given in Table 1.

There was a marked seasonal effect superimposed on the effect of chlorine level on amino acid concentration. Harvest times for the three experiments reported upon were October, January, and March respectively.

A comparison of amino acid concentrations found in plus-chloride cabbage with those of the chloride-deficient plants is given in Table 2. Comparable results pertaining to cauliflower are given in Table 3. Only the mean values for the three experiments are given.

Restrictions in chloride supply to the two species were manifested by large quantitative differences in amino acid composition, but qualitative effects were closely comparable. Most striking is the comparatively large increase in the concentrations of arginine, proline, methionine sulphoxide, glutamine, asparagine, and pipecolic acid observed under conditions of chloride restriction, particularly in cabbage.

Accumulation of arginine in plant tissue has been reported to result from deficiencies of other essential elements. Richards and Berner (1954) found an increase in this amino acid in potassium-deficient barley. Similarly, Holley and Cain (1955) found a higher concentration of arginine in the leaves of apple, blueberry, and magnolia plants affected by an iron deficiency than in leaves of normal plants. Mertz, Singleton, and Garey (1952) and Coleman (1957) found similar increases in arginine in sulphur-deficient plants. Hewitt, Jones, and Williams (1949) observed that a manganese deficiency in cauliflower increased the arginine concentration. Possingham (1956) reported that a deficiency of zinc resulted in a statistically significant increased arginine concentration. Gleiter and Parker (1957) obtained large increases in arginine with phosphorus-deficient alfalfa.

Table 1

VARIATION OBTAINED WITHIN AND BETWEEN EXPERIMENTS FOR THE ASPARAGINE AND α-ALANINE CONCENTRATIONS IN CAULIFLOWER PLANTS

Concentrations expressed as μg/g fresh tissue

Amino Acid	First Experiment		Second Experiment		Third Experiment	
	+Chloride	−Chloride	+Chloride	−Chloride	+Chloride	−Chloride
Asparagine	138	263	134	293	57	134
	139	239	124	319	65	136
	140	246	140	297	67	140
α-Alanine	132	200	104	176	79	289
	137	174	119	174	91	250
	134	188	106	166	82	272

Mertz and Matsumoto (1956) suggested that the accumulation of arginine in sulphur-deficient plants may be related to the metabolism of aspartic and glutamic acids. The close metabolic relationship of glutamic acid to arginine in plants has been discussed by Coleman and Hegarty (1957) and Kasting and Delwiche (1958).

The accumulation of proline observed under conditions of a chloride deficiency has not beeen reported for other nutrient deficiencies. Steinberg (1956) reported a decrease in proline with deficiencies of molybdenum, iron, boron, copper, zinc, and manganese. Possingham (1956) detected a small increase in proline with copper deficiency but no significant differences between zinc-, manganese-, iron-, or molybdenum-deficient and -sufficient plants. Gleiter and Parker (1957) found a decrease in proline with a phosphorus deficiency. Coleman (1957) observed a similar depression with sulphur deficiency in the case of white clover and tomato, but a very small increase with barley in one experiment.

In our experiments infiltration of uniformly labelled ^{14}C-glutamic acid into chloride-deficient and -sufficient cauliflower leaves, resulted in appreciable conversion of the glutamic acid to ^{14}C-labelled citrulline, arginine, and proline within 3 hr, with no significant difference due to chloride starvation. The possibility of chlorine contamination in the infusion process cannot be ruled out. The labelled spots were detected by radioautographs of the chromatograms, and citrulline was identified by co-chromatography with authentic citrulline. Citrulline was not present in sufficient quantity in chloride-deficient or -sufficient plant material to permit quantitative determination, and ornithine was not detected in any of the extracts.

TABLE 2

EFFECT OF CHLORINE STATUS ON THE CONCENTRATIONS OF FREE AMINO ACIDS IN CABBAGE PLANTS

Amino Acid	μg Amino Acid/g Fresh Tissue		Percentage Change
	+Chloride	−Chloride	
Arginine	<1	113	>11,200**
Proline	3	150	4,900***
Methionine sulphoxide	<1	29	>2,800**
Glutamine	99	359	263***
Asparagine	36	124	244**
Pipecolic acid	9	29	222
Glycine	15	36	140*
Valine	21	49	133*
Leucine	18	41	128
Serine	63	138	119***
α-Alanine	71	154	117*
Ethanolamine	19	41	116
Threonine	32	67	109
γ-Aminobutyric acid	43	62	44
Glutamic acid	116	163	41
Aspartic acid	50	44	(−) 12

*Significant at the 10 per cent. level.
**Significant at the 5 per cent. level.
***Significant at the 1 per cent. level.

The accumulation of methionine sulphoxide under conditions of chlorine deficiency is unusual, in that the only other reported occurrence of a similar accumulation with a deficiency of an essential element is for copper in the case of tobacco (Steinberg 1956). However, Dent, Stepka, and Steward (1947) reported its occurrence in potato tubers, and Steward and Thompson (1950) observed its presence on chromatograms of extracts from a wide variety of plant tissues.

It should be noted that methionine sulphoxide in normal cabbage and cauliflower plants and arginine in normal cabbage plants were present only at the limit of detection, thus giving a very high apparent percentage increase in the concentration of these compounds in chloride-deficient material.

Prianischnikow (1922) showed that amides accumulate in plants during proteolysis. Thus, the increase in glutamine and asparagine with chlorine deficiency would suggest a disruption in protein synthesis. However, the data with regard to pipecolic acid and γ-aminobutyric acid, which are not normally considered to be constituents of protein, suggest that the results observed are not solely due to a suppression of protein synthesis. If the results reflect proteolysis or the failure to synthesize new protein, increases in the concentrations of amino acids which are not part of the protein could be due to a general shift in equilibria due to the change in concentration of a given amino acid. For example, the change in concentration of pipecolic acid could result from a shift in the concentrations of lysine or some other more remotely related amino acids.

TABLE 3

EFFECT OF CHLORINE STATUS ON THE CONCENTRATIONS OF FREE AMINO ACIDS IN CAULIFLOWER PLANTS

Amino Acid	μg Amino Acid/g Fresh Tissue		Percentage Change
	+Chloride	−Chloride	
Methionine sulphoxide	<3	27	>800**
Proline	32	227	609***
Pipecolic acid	9	40	344**
Arginine	29	115	297***
Glutamine	146	494	238**
Leucine	20	66	230**
Ethanolamine	33	85	158***
Valine	39	92	136***
Serine	86	189	120**
Asparagine	111	230	107**
α-Alanine	109	210	93
Threonine	50	86	72**
Glycine	24	35	46
γ-Aminobutyric acid	59	69	17
Glutamic acid	149	162	9
Aspartic acid	60	64	7

*Significant at the 10 per cent. level.
**Significant at the 5 per cent. level.
***Significant at the 1 per cent. level.

On the basis of the data discussed above, it can be concluded that the chloride status of the plant can have a profound effect upon the concentrations of some amino acids in its tissues. The effect is a complicated and variable one dependent upon the physiological age of the plants, and the species studied, as well as upon the degree of chloride starvation, and undoubtedly other factors. Particularly large effects were noted in the case of arginine, proline, pipecolic acid, and methionine sulphoxide. The effects observed probably are not due to a direct effect of chloride ion on the interconversion or synthesis of amino acids but more likely are a manifestation of the disruption of some other reaction to which the amino acids are only indirectly related.

IV. Acknowledgments

This work was carried out at the Kearney Foundation of Soil Science, University of California, during the tenure, by J. R. Freney, of a C.S.I.R.O. Studentship. We are indebted to Mr. P. F. May, Division of Mathematical Statistics, C.S.I.R.O., for statistical treatment of the data.

V. References

Archer, R., Fromageot, C., and Jutisz, M. (1950).—*Biochem. Biophys. Acta* **5**: 81–8.

Berry, H. K., and Cain, L. (1949).—*Arch. Biochem.* **24**: 179–89.

Broyer, T. C., Carlton, A. B., Johnson, C. M., and Stout, P. R. (1954).—*Plant Physiol.* **29**: 526–32.

Coleman, R. G. (1957).—*Aust. J. Biol. Sci.* **10**: 50–6.

Coleman, R. G., and Hegarty, M. P. (1957).—*Nature* **179**: 376–7.

Decker, P., and Riffart, W. (1950).—*Chem. Ztg.* **74**: 261.

Dent, C. E., Stepka, W., and Steward, F. C. (1947).—*Nature* **160**: 682–3.

Gleiter, M. E., and Parker, H. E. (1957).—*Arch. Biochem. Biophys.* **71**: 430–6.

Hewitt, E. J., Jones, E. W., and Williams, A. H. (1949).—*Nature* **163**: 681–2.

Holley, R. W., and Cain, J. C. (1955).—*Science* **121**: 172–3.

Johnson, C. M., Stout, P. R., Broyer, T. C., and Carlton, A. B. (1957).—*Plant & Soil* **8**: 337–53.

Kasting, R., and Delwiche, C. C. (1958).—*Plant Physiol.* **33**: 350–4.

Mertz, E. T., Singleton, V. L., and Garey, C. L. (1952).—*Arch. Biochem. Biophys.* **38**: 139–45.

Mertz, E. T., and Matsumoto, H. (1956).—*Arch. Biochem. Biophys.* **63**: 50–63.

Ozanne, P. G., Woolley, J. T., and Broyer, T. C. (1957).—*Aust. J. Biol. Sci.* **10**: 66–79.

Possingham, J. V. (1956).—*Aust. J. Biol. Sci.* **9**: 539–51.

Prianischnikow, D. (1922).—*Ber. dtsch. bot. Ges.* **40**: 242–8.

Reed, L. J. (1950).—*J. Biol. Chem.* **183**: 451–8.

Richards, F. J., and Berner, E. (1954).—*Ann. Bot.* (N.S.) **18**: 15–33.

Sakaguchi, S. (1950).—*J. Biochem., Japan* **37**: 231–6.

Smith, E. L., and Tuller, E. F. (1955).—*Arch. Biochem. Biophys.* **54**: 114–20.

Steinberg, R. A. (1956).—In "Inorganic Nitrogen Metabolism". pp. 153–8. (Ed. W. D. McElroy and B. Glass.) (The Johns Hopkins Press: Baltimore.)

Steward, F. C., and Thompson, J. F. (1950).—*Annu. Rev. Pl. Physiol.* **1**: 233–64.

Toennies, G., and Kolb, J. J. (1951).—*Anal. Chem.* **23**: 823–6.

Ulrich, A., and Ohki, K. (1956).—*Plant Physiol.* **31**: 171–81.

Warburg, O., and Lüttgens, W. (1946).—*Biochimia* **11**: 303–21.

Wellington, E. F. (1952).—*Canad. J. Chem.* **30**: 581–91.

OXIDATION OF KREBS CYCLE ACIDS BY TISSUE SLICES AND CYTOPLASMIC PARTICLES FROM APPLE FRUIT

By M. D. Hatch,* Judith A. Pearson,† Adele Millerd,‡ and R. N. Robertson†

[*Manuscript received November 24, 1958*]

Summary

It has previously proved difficult to demonstrate the Krebs cycle in either cytoplasmic particles or tissue slices obtained from apple fruit. In the present investigation, evidence was obtained for the operation of the classical Krebs cycle–cytochrome oxidase respiratory system in cut tissue and mitochondria from Granny Smith apples. The respiration of cut tissue increased when either citrate, α-ketoglutarate, succinate, malate, fumarate, or pyruvate were added. Both the endogenous and acid-stimulated respiration were inhibited by malonate, cyanide, and azide. The rapid oxidation of Krebs cycle acids by cytoplasmic particles from apple flesh was also demonstrated. These particles showed cytochrome oxidase activity and contained a cytochrome *c*-dependent succinoxidase system.

I. Introduction

Contrary to earlier evidence (Hackney 1949; Webster 1952, 1954) cytochrome oxidase was found to mediate most of the respiration of mature apple fruits (Nakabayashi 1954; Rubin and Ladygina 1956). Cytochrome oxidase activity has also been identified in particulate preparations obtained from apple tissue (Rubin and Ladygina 1956; Lieberman 1958). These findings suggest that apple respiration is mediated by a Krebs cycle–cytochrome oxidase system typical of many other plant tissues. However, the operation of such a system has been difficult to demonstrate (Hulme 1958). There have been numerous reports which describe the effects of organic acids on the respiration of apple tissue (Hackney 1947; Porritt 1951; Siegelman and Schomer 1954; Neal and Hulme 1958*a*). Although some stimulatory effects were observed, in general the acids either had no effect on respiration or were inhibitory. In no case was respiration increased by all the acids of the Krebs cycle.

Difficulty has also been encountered in demonstrating the oxidation of Krebs cycle acids by apple fruit mitochondria (Neal and Hulme 1958*b*; Lieberman, personal communication), although Pearson and Robertson (1954) have reported that succinate, citrate, and malate caused some increase in oxygen uptake by a particulate preparation from Granny Smith apples. Neal and Hulme (1958*b*) also

---

* Biochemistry Department, University of Sydney; present address: Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., and Botany School, University of Sydney.

† Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., and Botany School, University of Sydney.

‡ Biochemistry Department, University of Sydney; present address: Waite Agricultural Research Institute, University of Adelaide.

showed that succinate and malate were oxidized by an apple particulate preparation supplemented with substrate levels of protocatechuic acid. Polyphenol oxidase was implicated in this oxidation process.

The present report provides evidence for the operation of a Krebs cycle–cytochrome oxidase system in tissue slices and mitochondria obtained from Granny Smith apples.

II. Materials and Methods

(a) *Materials*

Experiments were carried out on the cortex tissue of Granny Smith apples obtained from one orchard. Except where otherwise indicated the apples used were commercially mature and had entered the climacteric phase. As previously observed (Pearson and Robertson 1954) the climacteric rise of respiration commenced at approximately 190 days from full blossom.

Table 1

EFFECT OF pH ON THE OXIDATION OF KREBS CYCLE ACIDS BY APPLE TISSUE

Reaction mixtures contained 1 g of tissue slices in a solution containing sucrose (0·45M), phosphate buffer (0·01M) (pH as indicated), and substrate (0·04M) as indicated. Solutions of organic acids were previously adjusted to the appropriate pH. Pre-climacteric fruit (150 days from full blossom) was used in this experiment. Results are expressed as the percentage increase in respiration compared with endogenous rates

pH of Reaction	Substrate					
	Succinate	Citrate	Malate	α-Keto-glutarate	Fumarate	Pyruvate
3	98	—	34	—	—	—
4	156	36	36	—	—	—
5	117	25	50	61	29	50
6	123	29	28	—	40	44
7	77	33	30	33	17	22

(b) *Methods and Reagents*

Oxygen uptake was measured at 30°C by the standard Warburg manometric technique. The total nitrogen content of mitochondrial suspensions was determined by Nesslerization following acid digestion. Spectroscopic examination of mitochondrial preparations was carried out with a Zeiss-Winkel hand spectroscope.

Phosphate buffers contained potassium dihydrogen phosphate and disodium hydrogen phosphate. Yeast coenzyme (Le Page and Mueller 1949) and cytochrome *c* (Keilin and Hartree 1937) were prepared in the laboratory. Cytochrome *c* was standardized as described by Umbreit, Burris, and Stauffer (1951). Adenosine monophosphate and sodium pyruvate were obtained from L. Light & Co., England.

(c) *Application of Inhibitors*

Cyanide-inhibition experiments were conducted with calcium hydroxide–calcium cyanide mixtures in the centre well of Warburg vessels to absorb carbon dioxide (Robbie and Leinfelder 1945; Robbie 1948). Malonate was added with substrate at zero time and its effect on respiration, compared with control experiments, was measured after 1 hr.

(d) *Assay of Succinoxidase and Cytochrome Oxidase*

Cytochrome *c*-dependent succinoxidase and cytochrome oxidase activity were assayed according to Umbreit, Burris, and Stauffer (1951). *p*-Phenylenediamine was used instead of ascorbic acid to reduce cytochrome *c* (Maxwell 1950).

(e) *Preparation of Cut Tissue*

Disks 1 mm thick were cut from cylinders of the fruit removed with a cork borer. The subsequent treatment of these disks varied. Disks used in the experiments described in Table 1 were weighed into 1-g lots, placed in a small piece of muslin, and rinsed in a solution of sucrose (0·45M) and potassium dihydrogen phosphate (0·01M). Disks used in the experiments described in Tables 2 and 3 were washed three times in a solution of potassium dihydrogen phosphate (0·15M), then blotted dry between filter paper and weighed out into 1·5-g (±0·05 g) lots.

(*f*) *Preparation of Cytoplasmic Particles*

(i) *Cell-free Particulate Extract.*—The particulate fraction used in the experiments of Table 4 was prepared as previously described (Pearson and Robertson 1954).

(ii) *Mitochondria.*—100 g of apple flesh was cut into small pieces and blended with 65 ml of a solution containing phosphate buffer (0·1M) (pH 7·4), sucrose (0·5M), and ethylenediaminetetra-acetic acid (EDTA) (2×10^{-3}M). During the blending the pH was kept approximately neutral by the addition of 5 per cent. potassium hydroxide. The homogenate was strained through three layers of fine muslin and the filtrate centrifuged at 2000 *g* for 5 min. The supernatant was then centrifuged at 16,000 *g* for 10 min. The residue was resuspended in a solution containing sucrose (0·5M) and EDTA (2×10^{-3}M) and centrifuged at 16,000 *g* for 10 min. This residue was suspended in a solution containing sucrose (0·5M) and phosphate buffer (0·1M) (pH 7·1) and aliquots were used for manometric studies.

The particulate preparations used for the assay of succinoxidase and cytochrome oxidase activity were prepared as mitochondria except that the particles were washed with distilled water instead of the sucrose–EDTA solution.

III. Results

(a) *Effect of pH on the Respiration of Organic Acids by Apple Slices*

It is well known that pH affects the penetration of ionizable substances into living cells. The results of Table 1 show that the increase of apple slice respiration resulting from the addition of Krebs cycle acids varied between 20 and 150 per cent.

depending upon the individual acid and the pH employed. Different acids produced maximal stimulation at different pH values. Since the addition of 0·03M NaCl caused no appreciable increase in respiration under similar conditions it was concluded that the results with acids were not due to unspecific salt effects. Only small changes in the rate of endogenous respiration were observed over the pH range employed in this experiment.

In separate experiments it was shown that the respiratory quotient (R.Q.) of cut tissue was increased by the addition of organic acids. When succinate and malate were added the R.Q. was increased from 1·5 to 1·9 and 2·8 respectively.

Table 2

MALONATE INHIBITION OF ENDOGENOUS AND KREBS CYCLE ACID-STIMULATED APPLE SLICE RESPIRATION

Reaction mixtures contained 1·5 g of tissue slices in 0·15M potassium dihydrogen phosphate (pH 4·5). Substrates (10^{-2}M) and malonate (2×10^{-2}M) were added as indicated. Results expressed as rate of oxygen consumption (μl/hr/flask)

Additions	Substrate				
	None	Succinate	Citrate	α-Keto-glutarate	Malate
Substrate	31	57	44	50	55
Substrate plus malonate	16	18	16	21	20

(b) *Inhibition of Respiration of Apple Slices*

(i) *Malonate.*—The endogenous respiration of apple slices was inhibited by almost 50 per cent. by 0·02M malonate (Table 2). However, the increased respiration due to added Krebs cycle acids was completely abolished by the same concentration of the inhibitor. These results implicate, succinic dehydrogenase, in the endogenous and acid-stimulated respiration of apple tissue.

(ii) *Cyanide, Azide, and Phenylthiourea.*—The metal-binding agents cyanide and azide almost completely inhibited endogenous and succinate-stimulated respiration (Table 3). In separate experiments 4×10^{-4}M phenylthiourea, which inhibits copper-dependent oxidases (Bonner 1957), had no effect on apple slice respiration. Under similar conditions, however, this reagent completely inhibited the oxidation of *p*-cresol by the tissue slices. These results provided indirect evidence for the operation of cytochrome oxidase rather than a copper-containing oxidase in the normal respiration of the tissue.

(c) *Respiratory Activity of an Apple Particulate Extract*

It was previously established that oxygen uptake by an unwashed particulate extract from Granny Smith apple tissue was increased by the addition of succinate, malate, and citrate (Pearson and Robertson 1954). These results were confirmed and extended (see Table 4). Added α-ketoglutarate and pyruvate increased the respiration of particles from both pre-climacteric and climacteric tissue. As previously observed, a marked increase of endogenous oxygen uptake and a smaller increase in the rate of acid oxidation by the particulate preparation was closely associated with the onset of the climacteric rise of whole fruit respiration. In unreported experiments the R.Q. of the particulate extract in the absence of substrate was found to be 1·3. This suggested that the observed oxygen uptake was due to the metabolism of organic acids.

Table 3

CYANIDE AND AZIDE INHIBITION OF ENDOGENOUS AND SUCCINATE-STIMULATED APPLE SLICE RESPIRATION

Reaction mixtures as in Table 2. Cyanide and azide were added to give a final concentration of 2×10^{-3}M

Additions	Respiration (μl O_2/hr/flask)	Additions	Respiration (μl O_2/hr/flask)
None	23	Succinate	45
Cyanide	2	Succinate + cyanide	4
Azide	0	Succinate + azide	0

Table 4

RESPIRATORY ACTIVITY OF PARTICULATE FRACTIONS FROM PRE-CLIMACTERIC AND CLIMACTERIC APPLE TISSUE

Reaction mixtures contained 1 ml of the particulate suspension, phosphate buffer pH 7·0 (0·01M), $MgSO_4$ (10^{-3}M), and substrate (0·02M) as indicated

Tissue Age (days from full blossom)	Oxygen Consumption (μl/mg N/hr)						
	Substrate						
	None	Succinate	Citrate	Malate	α-Ketoglutarate	Fumarate	Pyruvate
150	10	25	15	15	17	16	28
210	35	58	62	46	50	36	50

(d) *Oxidation of Krebs Cycle Acids by Cytoplasmic Particles Prepared as Mitochondria*

Particles prepared from apple tissue by the standard techniques for the isolation of mitochondria oxidized Krebs cycle acids at a relatively rapid rate

(Table 5). The rate of endogenous oxygen consumption by the washed particles was very low.

(*e*) *Succinoxidase and Cytochrome Oxidase Activity of Disintegrated Mitochondria*

The cytochrome *c*-dependent oxidation of succinate by water-treated mitochondria was demonstrated (Table 6). An increase of 140 per cent. in the rate of succinate oxidation was observed upon the addition of cytochrome *c*. These results indicated that a typical cytochrome oxidase-mediated succinoxidase system was operating. Cytochrome *c* bound to the fragmented mitochondria may have supported the remainder of the succinate oxidation. Cytochrome oxidase activity was directly demonstrated using *p*-phenylenediamine to reduce cytochrome *c* (Table 6). Similar results were obtained when *p*-phenylenediamine was replaced by ascorbic acid.

TABLE 5

OXIDATION OF KREBS CYCLE ACIDS BY MITOCHONDRIA FROM APPLE TISSUE

Reaction mixtures contained mitochondrial suspension (0·3–0·35 mg N); sucrose (0·5M); phosphate buffer pH 7·0 (0·05M); adenosine monophosphate (10^{-3}M); $MgCl_2$ (10^{-3}M), and 0·1 per cent. yeast coenzyme. Substrate (0·02M) was added as indicated

Experiment	Oxygen Consumption (μl/mg N/hr)				
	Substrate				
	None	Succinate	α-Ketoglutarate	Citrate	Malate
1	10	124	130	80	—
2	16	210	176	—	195

When mitochondrial suspensions were treated with hydrosulphite the appearance of an absorption band at 563 mμ was observed. This corresponds to the α-absorption band of a reduced *b*-type cytochrome. Apparently the concentrations of cytochrome oxidase and cytochrome *c* were not sufficient to be detected by this method.

IV. DISCUSSION

Quantitative data relating changes in the respiration of apple fruits to the processes of maturation and senescence have been obtained previously (Robertson and Turner 1951; Pearson and Robertson 1952, 1953, 1954). However, a more complete understanding of the significance of the climacteric rise of respiration, and the processes by which cold storage and high carbon dioxide concentrations prolong the onset of senescence, require further knowledge of the mechanism of respiration in apple fruits. The results of the present investigation provide evidence for the operation of a Krebs cycle–cytochrome oxidase system in apple tissue.

Malonate inhibition of succinate oxidation by apple tissue was reported by Hackney (1947), indicating that succinic dehydrogenase was operating. In the present investigation the increase in respiration caused by the addition of other Krebs cycle acids was also inhibited by malonate. It was concluded that each of these acids was oxidized by a pathway involving succinic dehydrogenase. Malonate also inhibited endogenous oxygen consumption of apple slices by about 50 per cent. This observation could be interpreted in one of two ways. Either a pathway other than the Krebs cycle mediated the malonate-insensitive respiration, or the competing power of malonate for the active sites on succinic dehydrogenase was insufficient at the concentration used to cause complete inhibition.

TABLE 6

SUCCINOXIDASE AND CYTOCHROME OXIDASE ACTIVITY OF DISINTEGRATED MITOCHONDRIA

Reaction mixtures contained the particulate suspension in phosphate buffer pH 7·0 (0·1M). Succinate (5×10^{-2}M), *p*-phenylenediamine (10^{-2}M), and cytochrome *c* (2×10^{-5}M) were added as indicated

Experiment 1		Experiment 2	
Additions	Oxygen Consumption (μl/mg N/hr)	Additions	Oxygen Consumption (μl/hr/flask)
None	0	None	6
Succinate	53	Cytochrome *c*	4
Succinate + cytochrome *c*	126	*p*-Phenylenediamine	43
		p-Phenylenediamine + cytochrome *c*	146

It was shown that pH had a specific and marked affect upon the rates at which organic acids were respired by tissue slices. This may have been associated with the pK values for the carboxyl groups of individual acids. pH could have been one of the factors responsible for the variable results previously reported on the effects of organic acids on the respiration of apple tissue.

Earlier evidence suggested that either polyphenol oxidase or ascorbic acid oxidase mediated apple respiration (Hackney 1949). Subsequently Nakabayashi (1954) showed that apple respiration was almost completely inhibited by carbon monoxide and that this inhibition was reversed by light. This finding indicated that cytochrome oxidase was the only important respiratory terminal oxidase. Evidence in the present investigation supported this conclusion. Cyanide and azide almost completely inhibited the endogenous and succinate-stimulated respiration of apple slices. However, endogenous respiration was not affected by phenylthiourea which binds copper and hence inhibits polyphenol oxidase and ascorbic acid oxidase.

This reagent completely inhibited the oxidation of added *p*-cresol which was presumably mediated by polyphenol oxidase.

The Krebs cycle–cytochrome oxidase system, which apparently operated in cut tissues from Granny Smith apples, is generally associated with the mitochondrial fraction of cells. Though difficulty has been encountered in demonstrating this system in mitochondrial preparations from apple tissue (Neal and Hulme 1958*b*; Lieberman, personal communication), in the present investigation a particulate fraction, prepared by the usual procedure for mitochondria, was found to oxidize Krebs cycle acids. The fraction was also shown to contain the cytochrome oxidase and cytochrome *c*-dependent succinoxidase activities characteristic of mitochondria obtained from other tissues.

V. Acknowledgments

This work was carried out partly in the Department of Biochemistry, University of Sydney, and partly in the Plant Physiology Unit of the Botany School, University of Sydney, and of the Division of Food Preservation and Transport, C.S.I.R.O.

The authors wish to express thanks to Professor J. L. Still, Department of Biochemistry, University of Sydney, Professor R. L. Crocker, Botany School, University of Sydney, and Dr. R. J. Vickery, Chief, Division of Food Preservation and Transport, C.S.I.R.O., in whose laboratories the work was carried out, and to Dr. F. E. Huelin and Dr. J. H. B. Christian for helpful criticism of the manuscript.

VI. References

Bonner, W. D. (1957).—*Annu. Rev. Pl. Physiol.* **8**: 427.

Hackney, F. M. V. (1947).—D.Sc. Thesis, University of Sydney.

Hackney, F. M. V. (1949).—*Proc. Linn. Soc. N.S.W.* **73**: 439, 455.

Hulme, A. C. (1958).—*Advanc. Food. Res.* **8**: 297.

Keilin, D., and Hartree, E. F. (1937).—*Proc. Roy. Soc.* B **122**: 298.

Le Page, G. A., and Mueller, G. C. (1949).—*J. Biol. Chem.* **180**: 975.

Lieberman, M. (1958).—*Science* **127**: 189.

Maxwell, R. E. (1950).—*Plant Physiol.* **25**: 521.

Nakabayashi, T. (1954).—*J. Agric. Chem. Soc., Japan* **28**: 212.

Neal, G. E., and Hulme, A. C. (1958*a*).—*J. Exp. Bot.* **9**: 142.

Neal, G. E., and Hulme, A. C. (1958*b*).—*Nature* **181**: 1469.

Porritt, S. W. (1951).—Annu. Rep. Fruit Veg. Prod. Res. Comm. Canad. Dep. Agric.

Pearson, J. A., and Robertson, R. N. (1952).—*Aust. J. Sci.* **15**: 99.

Pearson, J. A., and Robertson, R. N. (1953).—*Aust. J. Biol. Sci.* **6**: 1.

Pearson, J. A., and Robertson, R. N. (1954).—*Aust. J. Biol. Sci.* **7**: 1.

Robbie, W. A. (1948).—*Meth. Med. Res.* **1**: 307.

Robbie, W. A., and Leinfelder, P. J. (1945).—*J. Industr. Hyg.* **27**: 269.

Robertson, R. N., and Turner, J. F. (1951).—*Aust. J. Sci. Res.* B **4**: 92.

Rubin, B. A., and Ladygina, M. E. (1956).—*Biochemistry, Leningr.* **21**: 347.

Siegelman, H. W., and Schomer, H. A. (1954).—*Plant Physiol.* **29**: 429.

Webster, G. C. (1952).—*Amer. J. Bot.* **39**: 739.

Webster, G. C. (1954).—*Plant Physiol.* **29**: 399.

Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1951).—"Manometric Techniques and Tissue Metabolism." (Burgess Publ. Co.: Minneapolis.)

STUDIES IN THE NATURAL COATING OF APPLES

IV. THE NATURE OF CUTIN

By F. E. Huelin*

[*Manuscript received October* 13, 1958]

Summary

Cutin purified with snail gut extract yielded, on alkaline hydrolysis, ether-soluble acids, a water-soluble fraction containing carbohydrates, and an insoluble fraction containing protein. A number of sugars and amino acids were identified in the acid hydrolysates of the last two fractions.

No evidence of continuing polymerization of the cutin during storage was obtained.

It is suggested that cutin is a polyestolide of hydroxy acids and other substances. Possible modes of formation are discussed.

I. Introduction

Cutin is the fraction of plant cuticles which is insoluble in water and organic solvents. In previous work on the natural coating of apples (Huelin and Gallop 1951*a*) the cutin was obtained mixed with epidermal cell wall material by separating the skin with acid ammonium oxalate solution and extracting it successively with light petroleum, ethyl ether, and ethanol. From this impure cutin about 50 per cent. of ether-soluble acids was obtained by boiling with 0·5N ethanolic potassium hydroxide for 2 hr. These acids gave the following characteristics: acid number 150–180, hydroxyl number 208, iodine number (Wijs) 50. The identification of the acids from apple cutin has not been attempted, but the cutin of *Agave americana* L. has been found by Matic (1956) to yield mono-, di-, and trihydroxy-C_{18} acids and a dihydroxy-C_{16} acid, each with one hydroxyl in the ω-position.

Huelin and Gallop (1951*a*) were unable to determine whether cutin yields other substances besides the ether-soluble acids. Ethanolic potassium hydroxide removed the cuticle completely from a microscopic section, but this result could indicate breakdown of structure rather than complete chemical solution. Purification of the cutin is essential, and it is now known that the cuprammonium solution used by Huelin and Gallop (1951*a*) fails to dissolve completely the epidermal cell walls.

II. Purification of Cutin

The mixture of enzymes contained in snail gut extract was used to dissolve the epidermis. The procedure was similar to that of Bawden and Pirie (1946). The snail crops were blended with a minimum volume of water and the extract centrifuged. Granny Smith apple skin, composed of cuticle and epidermis, was extracted with solvents and cut into small pieces approx. 0·5 cm² in area. These

*Division of Food Preservation and Transport, C.S.I.R.O., Homebush, N.S.W.

pieces were incubated for 7 days at 37°C with a 5 : 1 mixture of snail gut extract and 0·4M phosphate buffer of pH 7. Chloroform was used as antiseptic. The enzyme-treated pieces were washed thoroughly with water on a stainless steel screen. They were then washed with ethanol and dried. The purified cutin had a nitrogen content of 0·5 per cent.

Microscopic examination of sections of enzyme-treated skin (Plate 1, middle and bottom sections) showed that it was composed exclusively of cuticle, the epidermal cell walls being completely dissolved. The free cuticle coils spontaneously with the originally outer surface on the concave side. In contrast, treatment with cuprammonium solution, as previously carried out, did not remove the epidermis completely (Plate 1, top section).

III. Alkaline Hydrolysis of Cutin

The purified cutin yielded 77–83 per cent. of ether-soluble acids on treatment with 0·5N ethanolic potassium hydroxide. Initially, this involved boiling for 2 hr under reflux, but it was subsequently found that 98 per cent. of the total acid was liberated in 2 hr at 20°C. Saponification at 20°C in an atmosphere of nitrogen was adopted to ensure minimum degradation of the ethanol-insoluble products. After saponification the insoluble residue was filtered off, and the acids were recovered from the filtrate by evaporation of ethanol, dilution with water, acidification, and extraction with ether. Material extracted with ether before acidification was negligible.

The ethanol-insoluble residue was washed with ethanol containing 1 per cent. (v/v) acetic acid to dissolve any potassium carbonate, again washed with ethanol, and dried. It was then boiled with water for 5 min, filtered, and washed. The filtrate on evaporation yielded 7–14 per cent. of water-soluble fraction. The insoluble fraction (final residue) comprised 7–9 per cent. of the original cutin.

It appears that the complete removal of cuticle from a microscopic section after 2 weeks in 0·5N ethanolic potassium hydroxide at room temperature, which was previously reported (Huelin and Gallop 1951*a*), was due to solution of 90 per cent. of the cutin and disintegration of the residue. Before examination the treated section was stained in 70 per cent. (v/v) aqueous ethanol, and this has been shown to dissolve about half of the residue left after alkaline hydrolysis.

IV. Products of Alkaline Hydrolysis

(*a*) *Ether-soluble Acids*

The characteristics of the ether-soluble acids were similar to those previously reported. A typical sample gave the following analysis: acid number 167, hydroxyl number 255, iodine number 41, nitrogen 0·05 per cent. After more than 12 months at room temperature the acid number of this sample was only 92. This result is in accordance with the observation of Jensen (1950) that ω-hydroxy acids readily form estolides.

It has been shown that the acids liberated on saponification of solvent-extracted skin come exclusively from the cuticle and not from the epidermis. One sample of skin gave 59·9 per cent. of acids after saponification. A comparable weighed sample was incubated with snail gut extract and the residual cutin saponified to yield acids equivalent to 59·3 per cent. of the original skin.

(b) *Water-soluble Fraction*

The water-soluble fraction had a nitrogen content of 1·75 per cent. When the saponification was carried out at 20°C with liberation of 98 per cent. of the total acid, the remaining 2 per cent. was present in a combined form in the water-soluble fraction and was recovered by boiling with ethanolic potassium hydroxide for 2 hr.

The water-soluble fraction was hydrolysed by heating with 0·25N sulphuric acid at 100°C for 18 hr. This procedure was used by Gottschalk and Ada (1956) for the carbohydrate moiety of mucoproteins to minimize interaction between reducing sugars and amino acids. The precipitate formed during hydrolysis was equivalent to 22 per cent. of the original water-soluble fraction. To remove sulphuric acid the filtrate was neutralized with barium hydroxide to slight excess. After bubbling carbon dioxide the solution was again filtered. The soluble hydrolysate, which contained 0·73 per cent. of nitrogen (on a dry wt. basis), was examined by paper chromatography, using the procedure of Ash and Reynolds (1954) for sugars and the procedure of Reynolds (1957) for amino acids.

Arabinose, galacturonic acid, galactose, and ribose were detected and estimated approximately by comparison with reference spots. They comprised respectively 5, 2·5, 1·5, and 1 per cent. of the soluble hydrolysate. The first three substances indicate the presence of pectin in the water-soluble fraction. The ribose may be derived from nucleic acids or nucleotides. As amino acids were found to be negligible, unknown substances comprised about 90 per cent. of the water-soluble fraction.

(c) *Insoluble Fraction*

The insoluble fraction had a nitrogen content of 5·6 per cent., which accounted for three-quarters of the total nitrogen of the cutin. It was first hydrolysed with 0·25N sulphuric acid at 100°C for 18 hr, yielding 32 per cent. of soluble hydrolysate. A second hydrolysate, representing 39 per cent. of the insoluble fraction, was obtained by hydrolysing the residue with 6N hydrochloric acid at 110°C for 16 hr. The hydrochloric acid was removed by evaporating to dryness and holding in a vacuum desiccator over solid sodium hydroxide.

The first hydrolysate had a nitrogen content of 8·5 per cent. On paper chromatograms, aspartic acid appeared as the major component with traces of glutamic acid, serine, threonine, and alanine. The second hydrolysate contained 6·3 per cent. of nitrogen and the following amino acids: aspartic acid, glutamic acid, serine, threonine, alanine, valine, leucine or isoleucine or both, proline, glycine, tyrosine, phenylalanine, arginine, and histidine. The protein of Granny Smith apple flesh examined by McKee and Urbach (1953) contained all these amino acids except arginine and histidine. Hulme (1951) found arginine and Davis, Fellers, and Esselen (1949) found arginine and histidine in other varieties of apples.

V. CHANGES DURING STORAGE

Huelin and Gallop (1951*b*) obtained an increase of about 20 per cent. in the cutin fraction (as indicated by the yield of ether-soluble acids) during storage of Granny Smith apples.

The possibility of changes due to continuing polymerization of the cutin during storage was investigated. As polymerization is almost certainly due to the formation of ester linkages, it should be accompanied by a decrease in the concentration of free hydroxyl and carboxyl groups. Cutin from freshly picked apples and cutin from apples stored for 9 months at 1°C were examined by infra-red spectroscopy. No evidence of continuing polymerization was obtained (see Appendix I).

VI. DISCUSSION

The analysis of the fractions obtained on alkaline hydrolysis provides some information on the nature and origin of cutin. Hydrolysis under conditions associated with the fission of ester linkages produces about 80 per cent. of ether-soluble hydroxy acids together with water-soluble and insoluble fractions. It is plausible to regard the formation of the insoluble polymer as due primarily to the condensation of the constituent hydroxy acids by means of ester linkages. The carbohydrates and other unidentified compounds of the water-soluble fraction almost certainly participate in this condensation, as they become water soluble only after saponification. Direct evidence for the existence of ester linkages between the water-soluble constituents and the ether-soluble acids is provided by the fact that under certain conditions acids can be liberated from the water-soluble fraction. There is no evidence for the linkage of insoluble protein to the ether-soluble acids.

It appears that the formation of cutin is associated with the secretion of a great variety of substances by the tissue. Material secreted by the protoplasm and extruded through the cell wall would necessarily be in a soluble or disperse condition, and it is probable that the final change to the continuous layer of insoluble cutin occurs in the cuticle itself. The substances secreted may include soluble enzymes, which would subsequently be denatured and form part of the insoluble protein. The hypothesis of Lee and Priestley (1924), repeated by Priestley (1943), that unsaturated fatty acids oxidize spontaneously to form cutin on reaching the surface is unsatisfactory for the following reasons. These authors imply that oxidation is limited by access of oxygen except at the surface, whereas free oxygen is present throughout the tissue in the intercellular spaces and promotes other types of oxidation within the cells. Moreover, unsaturated lipids do not always oxidize spontaneously on reaching the surface; the unsaturated oil, which is secreted into the cuticle of apples during storage, shows no evidence of oxidation over a period of 6 months (Huelin and Gallop 1951*b*). It is more likely that the formation of cutin is under the control of specific and localized enzyme systems, although the final stage of condensation may be non-enzymic. Spontaneous condensation could most readily be restricted to the cuticle if the condensing units were produced *in situ*, although hydroxy acids may be formed within the cell in an environment unfavourable for condensation. The mechanism of formation of long-chain hydroxy acids, whether from the corresponding unsubstituted acids or from shorter units, is at present unknown.

The proportion of water-soluble material originally secreted into the cuticle may be much greater than that permanently retained, due to leaching by rain while the fruit is on the tree. The type of coiling obtained on separating the cuticle (with the outer surface on the concave side) is consistent with the view that the outer layers of cutin are formed first and become more stretched as the fruit grows.

VII. ACKNOWLEDGMENTS

The author is indebted to Dr. T. M. Reynolds for examining the acid hydrolysates by paper chromatography and identifying the sugars and amino acids; to Miss J. M. Bain for preparing sections of cuticle for microscopic examination; to Mr. J. J. Macfarlane for infra-red spectral examination of cutin; and to Dr. R. G. Wake, Biochemistry Department, University of Sydney, for determination of nitrogen.

VIII. REFERENCES

ASH, A. S. F., and REYNOLDS, T. M. (1954).—*Aust. J. Biol. Sci.* **7**: 435–43.

BAWDEN, F. C., and PIRIE, N. W. (1946).—*Brit. J. Exp. Path.* **27**: 81–90.

DAVIS, S. G., FELLERS, C. R., and ESSELEN, W. B. (1949).—*Food Res.* **14**: 417–28.

GOTTSCHALK, A., and ADA, G. L. (1956).—*Biochem. J.* **62**: 681–6.

HUELIN, F. E., and GALLOP, R. A. (1951*a*).—*Aust. J. Sci. Res.* B **4**: 526–32.

HUELIN, F. E., and GALLOP, R. A. (1951*b*).—*Aust. J. Sci. Res.* B **4**: 533–43.

HULME, A. C. (1951).—*J. Sci. Fd. Agr.* **2**: 160–6.

JENSEN, W. (1950).—*Finn. Pap. Timb.* B **32**: 261–6.

LEE, B., and PRIESTLEY, J. H. (1924).—*Ann. Bot., Lond.* **38**: 525–45.

MATIC, M. (1956).—*Biochem. J.* **63**: 168–76.

MCKEE, H. S., and URBACH, G. E. (1953).—*Aust. J. Biol. Sci.* **6**: 369–78.

PRIESTLEY, J. H. (1943).—*Bot. Rev., Lond.* **9**: 593–616.

REYNOLDS, T. M. (1957).—*Aust. J. Chem.* **10**: 198–202.

APPENDIX I

INFRA-RED SPECTRAL EXAMINATION OF CUTIN

By J. J. MACFARLANE*

The cutin was examined for evidence of any change in the number of OH or $C = O$ groups on storage.

Normally, quantitative absorption measurements are most conveniently carried out in solution, but in this case there appeared to be no suitable solvent. Owing to the nature of the cutin the potassium bromide disk technique could not be used. Hence a mulling method was adopted.

Hexachlorobutadiene was used as mulling agent since it has no absorption bands in the regions of interest. The absorptions due to the OH and $C = O$ groups were compared with those due to the CH_2 and CH_3 groups (which were assumed to be constant in cutin from freshly picked and stored apples). Each sample was examined over a range of cell thicknesses and log I_0/I (CH_2 and CH_3) plotted against log I_0/I ($C = O$) or log I_0/I (OH), the absorptions being measured at the peak maxima. As there is much scattering by the mull, I_0 at any particular absorption maximum was estimated by assuming that the background is given by a line joining points on the absorption curve lying just beyond the shoulders of the band. A change of OH or $C = O$ content relative to (CH_2 and CH_3) content will change the slope of the plot.

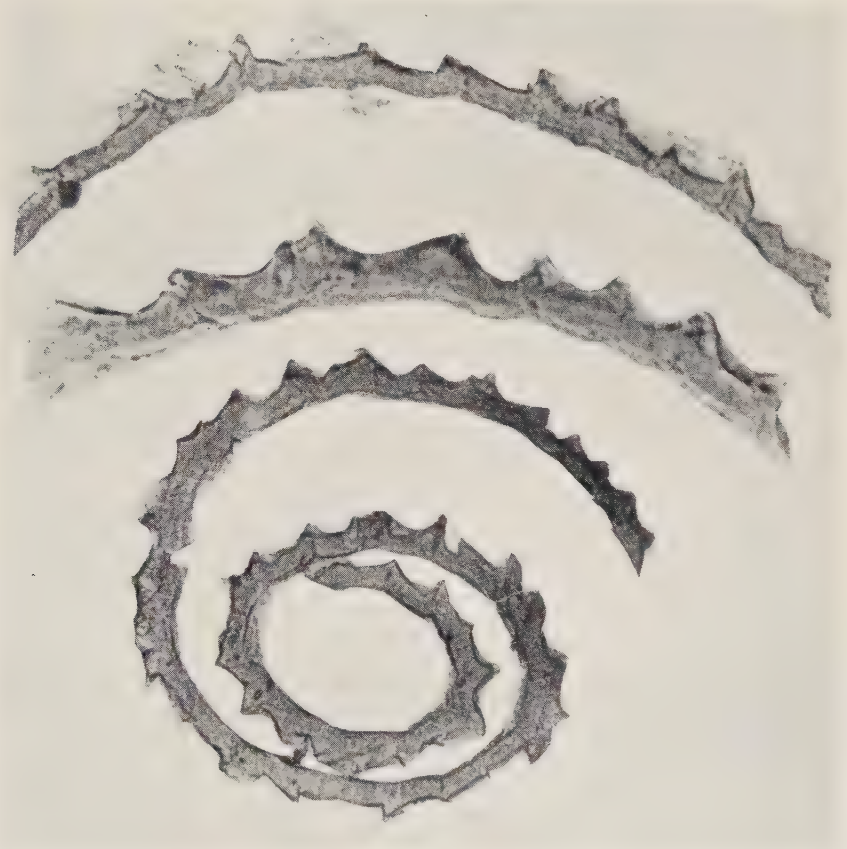
---

* Physicochemical Unit, Division of Food Preservation and Transport, C.S.I.R.O., Biochemistry Department, University of Sydney.

The plot of log I_0/I (OH) against log I_0/I (CH_2 and CH_3) was not linear, the deviations from linearity being sometimes large. Because of the uncertainty of the slope of the plot, only large changes in the relative OH concentration would be observed, probably of the order of 20–30 per cent.

Much better plots were obtained for log I_0/I ($C = O$) against log I_0/I (CH_2 and CH_3). From these it is estimated that a change in relative concentration of less than 10 per cent. would be detected.

No change in the relative OH group or $C = O$ group concentration could be shown from the plots obtained.



Top section: Skin treated with cuprammonium solution (×450).
Middle section: Skin treated with snail gut extract (×450).
Bottom section: Skin treated with snail gut extract (×200).

A DPNH-SPECIFIC NITRATE REDUCTASE FROM GERMINATING WHEAT

By D. Spencer*

[*Manuscript received October 26, 1958*]

Summary

The isolation and purification of a nitrate reductase from the embryos of germinating wheat is described. This is a soluble enzyme, which is coupled specifically to reduced diphosphopyridine nucleotide (DPNH). Addition of flavin adenine dinucleotide to the purified enzyme results in a three-fold increase in activity. Flavin mononucleotide is without effect. Potassium cyanide and sodium azide cause 50 per cent. inhibition of enzyme activity at $1 \cdot 6 \times 10^{-6}$M and 2×10^{-5}M respectively. The effect of a range of other inhibitors is reported. The presence of inorganic phosphate is required for maximum activity. The dissociation constant (K_s) of the nitrate–enzyme complex is $3 \cdot 8 \times 10^{-4}$M, and that of the DPNH–enzyme complex is 8×10^{-6}M. The pH optimum for enzymatic activity is $7 \cdot 4$.

A comparison is made with the published properties of the nitrate reductase from soybean leaves.

I. INTRODUCTION

Evans and Nason (1953) and Nicholas and Nason (1955*a*) have demonstrated that the nitrate reductase of soybean leaves is a molybdoflavoprotein which catalyses the reduction of nitrate to nitrite by either reduced di- or triphosphopyridine nucleotide (DPNH or TPNH). To date, this is the only nitrate reductase from higher plants which has been isolated and thoroughly characterized. The present report describes the isolation and properties of a nitrate reductase from the embryos of germinating wheat which, while being of the same general character, possesses some properties that are markedly different from those of the enzyme from soybean leaves.

II. MATERIALS AND METHODS

(*a*) *Isolation of the Enzyme*

Wheat seeds (*Triticum vulgare* var. Insignia) were germinated for 40–48 hr in darkness at 25°C on filter paper moistened with 10 mM potassium nitrate. At this stage the primary root was 10–15 mm long and the coleoptile 5–7 mm. The embryos were then dissected out at the junction with the scutellum, weighed, rinsed with water, and ground in potassium phosphate buffer. The endosperm was discarded since it contained no nitrate reductase and its presence made subsequent fractionation more difficult. The grindate was then centrifuged, and the crude, cell-free extract so obtained was subjected to ammonium sulphate fractionation. During isolation and purification the solutions were kept at 0–2°C, and all centrifugations were carried out at 5000 *g*. A buffer solution of $0 \cdot 1$M potassium phosphate, pH $7 \cdot 5$, which contained 10^{-3}M glutathione was used throughout.

---

* Division of Plant Industry, C.S.I.R.O., Canberra.

A typical isolation and purification was as follows: Approximately 7 g fresh weight of embryos (*c.* 700) were ground in a cold mortar with 80 ml of phosphate–glutathione solution, and the grindate was centrifuged for 20 min. The supernatant solution then comprised the crude, cell-free extract (vol. 80 ml). To this was added 28·1 g solid ammonium sulphate and, after standing 15–30 min, the precipitate was collected by centrifugation for 15 min. The precipitate was dissolved in phosphate–glutathione solution and the volume brought to 40 ml. This is the 0–55 per cent. ammonium sulphate fraction. To this was added 6·1 g solid ammonium sulphate, and after standing 15 min the precipitate was centrifuged down and discarded. The supernatant solution was treated with a further 3·2 g ammonium sulphate and again centrifuged. This precipitate, which is the 28–40 per cent. ammonium sulphate fraction, was dissolved in 10 ml of phosphate–glutathione solution and stored at $-15°C$. After thawing, the solution was diluted to 40 ml with phosphate–glutathione solution and brought to 29 per cent. saturation with 6·76 g ammonium sulphate. The precipitate so formed, which included clumped, denatured protein, was centrifuged down and discarded. A further 1·60 g ammonium sulphate were added to precipitate the 29–35 per cent. ammonium sulphate fraction which was centrifuged down and dissolved in 7 ml of phosphate–glutathione solution. Either this 29–35 per cent. ammonium sulphate fraction or the previous 28–40 per cent. fraction was used as the enzyme source throughout the experiments described here. A summary of the purification procedure and data on recovery and specific activity are given in Table 1. The final fraction showed a seven-fold increase in specific activity with a recovery of 58 per cent. of the activity of the crude, cell-free extract. The protein content of enzyme fractions was determined

TABLE 1

FRACTIONATION PROCEDURE FOR THE ISOLATION OF NITRATE REDUCTASE FROM GERMINATING WHEAT

Ammonium Sulphate Fraction	Volume (ml)	Total Nitrate Reductase Activity*	Percentage Recovery	Total Protein (mg)	Specific Activity†
Crude cell-free extract	80	8240	100	250	33
0–55 per cent.	40	6140	76	130	47
28–40 per cent.	10	7200	87	67	109
29–35 per cent.	7	4802	58	20	243

* Activity of nitrate reductase is expressed as mμmoles of nitrite formed in 30 min at 30°C in the standard assay (see Section II(*b*)).

† Specific activity expressed as mμmoles of nitrite formed per mg of protein in 30 min at 30°C.

by the method of Lowry *et al.* (1951) using crystalline bovine plasma albumin (Armour Laboratories) as a standard.

(*b*) *Assay Procedure*

Nitrate reductase activity was assayed as follows: 0·1M potassium nitrate (0·1 ml), 10^{-5}M flavin adenine dinucleotide (0·05 ml), 4×10^{-3}M DPNH (0·05 ml), nitrate reductase (0·05–0·20 ml, containing 100–250 μg of protein), and either 0·1M potassium phosphate, pH 7·5, or water to give a final volume of 0·5 ml were incubated for 30 min at 30°C, and the amount of nitrite formed was determined.

As has been reported by others (Evans and Nason 1953; Medina and Nicholas 1957) it was found that high residual concentrations of DPNH in the reaction mixture caused losses during the chemical determination of nitrite. This interference was obviated in two ways. At the end of the incubation period 0·05 ml of crystalline yeast alcohol dehydrogenase (0·75 mg/ml) and 0·05 ml of 0·1M acetaldehyde were added to the reaction mixture and incubation continued for a further 2 min. Then, as recommended by Medina and Nicholas (1957), molar barium acetate (0·1 ml) and ethanol (2·5 ml) were added. The suspension was centrifuged and nitrite was determined in the supernatant solution by the addition of 0·5 ml of 1 per cent. sulphanilamide in 2·5N HCl and 0·5 ml of *N*-(1-naphthyl)-ethylenediamine dihydrochloride (0·02 per cent. aqueous solution), and subsequent measurement of the optical density at 540 mμ in a Unicam S.P.600 spectrophotometer. Treatment with barium acetate and alcohol had the dual advantage of ensuring complete removal of residual DPNH and also of removing protein from the solution.

For the assay of crude cell-free extracts, a DPNH-generating system was used in the standard assay instead of DPNH. This consisted of 2×10^{-3}M diphosphopyridine nucleotide (DPN) (0·05 ml), 3M ethanol (0·05 ml), and 0·05 ml of crystalline yeast alcohol dehydrogenase (0·75 mg/ml). In this case the final treatment with acetaldehyde and alcohol dehydrogenase was omitted.

Under the above assay conditions, nitrate reductase activity was directly proportional to the time of incubation and to the enzyme concentration. Nitrate reductase activity is expressed in mμmoles of nitrite formed in 30 min at 30°C under the above standard assay conditions.

(*c*) *Special Reagents*

The DPN used was a gift from Dr. A. Nason of the McCollum-Pratt Institute, Johns Hopkins University, Baltimore. Triphosphopyridine nucleotide (TPN) (97 per cent. pure), flavin adenine dinucleotide (FAD) (31 per cent. pure), and flavin mononucleotide (FMN) were obtained from the Sigma Chemical Co., St. Louis. Crystalline yeast alcohol dehydrogenase was obtained from C. F. Boehringer & Son, Mannheim. *Iso*citric dehydrogenase was prepared from an acetone powder of pig heart (Ochoa 1955). The purification was carried through to the first ammonium sulphate fractionation. Trisodium *iso*citrate (*dl* and *allo*) was obtained from the California Foundation for Biochemical Research.

DPNH was prepared according to the procedure of Rafter and Colowick (1957), and TPNH as described by Evans and Nason (1953) using the *iso*citric dehydrogenase preparation described above.

III. Results

(*a*) *Pyridine Nucleotide Requirement*

Nitrate reductase isolated from young wheat embryos shows a specific and absolute dependence on added DPNH. No nitrite was formed in the absence of added DPNH or in the presence of TPNH (Fig. 1).

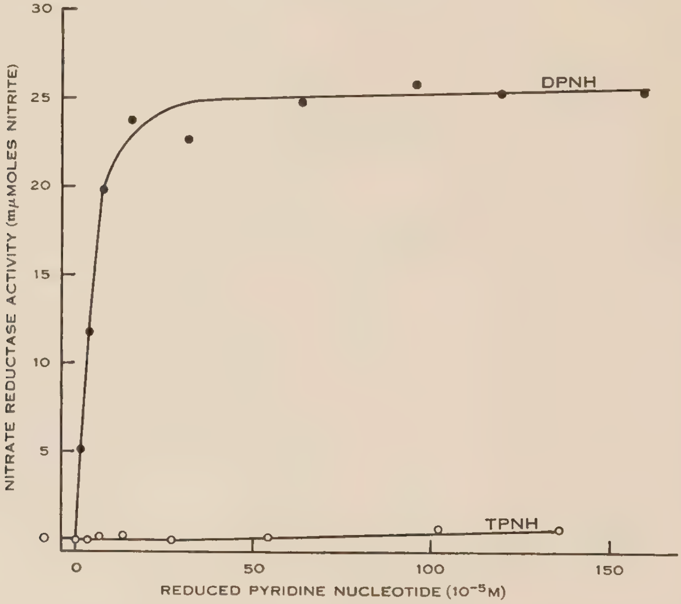


Fig. 1.—Effect of DPNH and TPNH on the activity of wheat embryo nitrate reductase. Enzyme activity is expressed in mμmoles of nitrite formed under conditions of standard assay, with varying DPNH and TPNH concentrations.

The enzyme was saturated with respect to DPNH at 4×10^{-4}M. The specific requirement for DPNH was confirmed by the use of systems which generated the reduced pyridine nucleotides in the assay. DPNH was generated from DPN by use of alcohol and alcohol dehydrogenase (see Section II(*b*)), and TPNH by means of TPN, *iso*citric dehydrogenase, and *iso*citrate (0·05 ml of 0·2M trisodium *iso*citrate, *dl* and *allo*).

The dissociation constant (K_S) of the DPNH–enzyme complex was calculated from values obtained when the DPN–alcohol dehydrogenase–ethanol system was used to generate a range of DPNH concentrations. It was established by parallel measurement of the optical density at 340 mμ that ethanol and alcohol dehydrogenase were in sufficient excess to keep the DPN fully reduced during the nitrate reductase assay. The K_s for the DPNH–enzyme complex was 8×10^{-6}M.

The effectiveness of both TPNH and the TPNH–*iso*citric dehydrogenase–*iso*citrate system as H-donors was checked using the nitrate reductase of soybean leaves, which is non-specific in its pyridine nucleotide requirement.

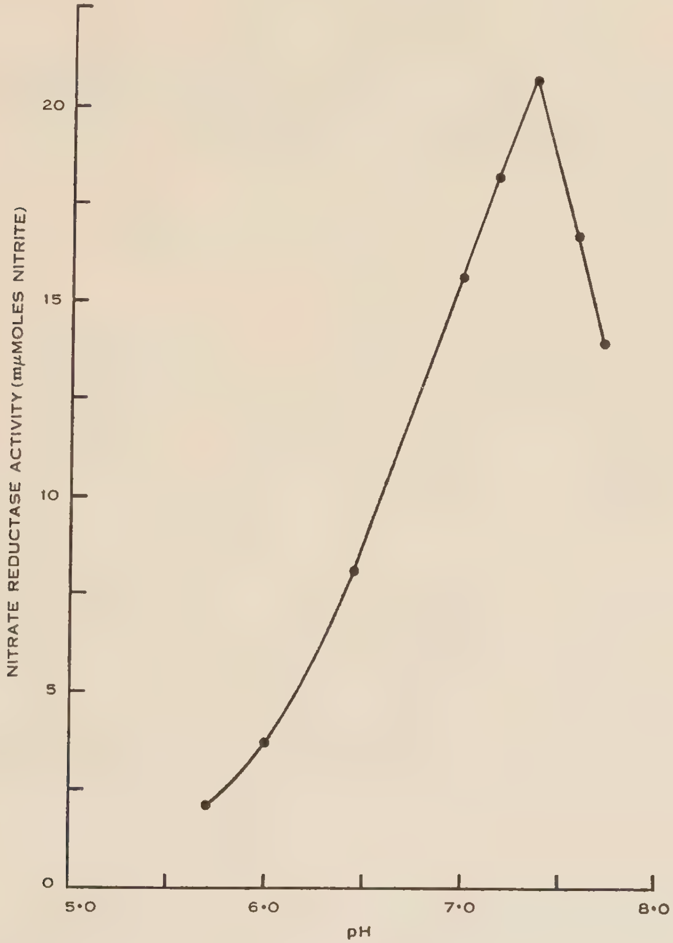


Fig. 2.—Effect of pH on the activity of wheat embryo nitrate reductase using phosphate buffers at a final concentration of 0·03M under standard assay conditions. The enzyme was dissolved in 0·1M potassium chloride solution and activity is expressed in mμmoles of nitrite formed under conditions of standard assay with varying pH.

(*b*) *pH Optimum*

The pH at which optimum nitrate reductase activity was obtained was 7·4 (Fig. 2). This was true whether DPNH as such or the DPN–alcohol dehydrogenase–ethanol system was used as the H-donor.

(c) *Flavin Requirement*

The nitrate reductase activity of crude cell-free extracts of embryos from germinating wheat was doubled by the addition of FAD. After isolation of the enzyme by ammonium sulphate fractionation, addition of FAD caused a three-fold stimulation at a final concentration of 10^{-6}M (Fig. 3). FMN was ineffective as a cofactor.

(d) *Nitrate Concentration for Optimum Activity*

Measurement of activity at a range of nitrate concentrations showed that the enzyme was saturated with respect to nitrate at 5×10^{-3}M. A boiled extract of an acetone powder of pig heart was used as the source of added flavin in this experiment since the FAD was found to be contaminated with nitrate. From the data so obtained the K_s of the nitrate–enzyme complex was calculated to be $3 \cdot 8\times10^{-4}$M.

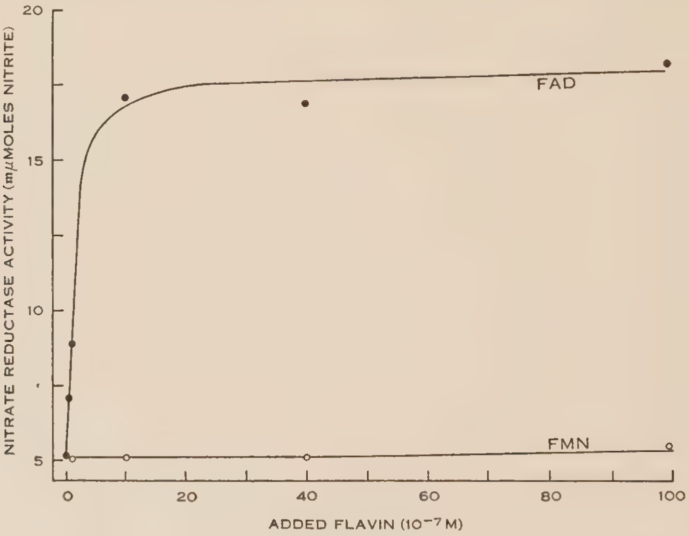


Fig. 3.—Effect of added FAD and FMN on the activity of wheat embryo nitrate reductase. Enzyme activity is expressed in mμmoles of nitrite formed under conditions of standard assay with varying FAD and FMN concentrations.

(e) *Inhibitors and Activators*

The enzyme was very sensitive to potassium cyanide and sodium azide, suggesting the requirement of a metal for enzymatic activity. Potassium cyanide caused 50 per cent. inhibition at $1 \cdot 6\times10^{-6}$M and sodium azide at 2×10^{-5}M. Other metal-binding agents tested were relatively ineffective. Inhibition by *p*-chloromercuribenzoate at low concentrations indicated a requirement for sulphydryl groups on the enzyme for catalytic activity. Nicholas (1958) has reported that the nitrite reductase of *Neurospora* is inhibited by uncoupling agents and that nitrite reduction is coupled to phosphate esterification. However, 2,4-dinitrophenol did not affect nitrate reductase activity up to a concentration of 2×10^{-4}M (Table 2).

It was not practicable to test the enzyme for inhibition by carbon monoxide since passage of a slow stream of either air, nitrogen, or carbon monoxide through a solution of the enzyme at 0°C for 5 min was sufficient to bring about complete inactivation, presumably due to surface denaturation.

Table 2

EFFECT OF A RANGE OF INHIBITORS ON THE ACTIVITY OF THE DPNH-SPECIFIC NITRATE REDUCTASE FROM GERMINATING WHEAT

Inhibitors were pre-incubated with the enzyme for 5 min at 0°C before testing their effect under standard assay conditions (see Section II(*b*))

Inhibitor	Final Concn. (M)	Percentage Inhibition
Potassium cyanide	10^{-4}	100
Potassium cyanide	10^{-5}	84
Potassium cyanide	10^{-6}	38
Sodium azide	10^{-3}	100
Sodium azide	10^{-4}	87
Sodium azide	10^{-5}	35
p-Chloromercuribenzoate	2×10^{-4}	100
p-Chloromercuribenzoate	2×10^{-5}	81
p-Chloromercuribenzoate	2×10^{-6}	0
8-Hydroxyquinoline	2×10^{-3}	8
a,*a*′-Dipyridyl	2×10^{-3}	0
o-Phenanthroline	2×10^{-3}	0
Ethylenediaminetetra-acetic acid	10^{-2}	0
Salicylaldoxime	2×10^{-3}	47
Allylthiourea	10^{-3}	0
2,4-Dinitrophenol	10^{-3}	4
Sodium orthovanadate	10^{-3}	100
Sodium orthovanadate	10^{-4}	77
Sodium orthovanadate	10^{-5}	32
Sodium orthovanadate	10^{-6}	13
Sodium tungstate	10^{-3}	0

Some slight enhancement of activity was found if enzyme extracts were prepared in the presence of 10^{-3}M glutathione. This was therefore routinely included in the isolation procedure. However, unlike the nitrate reductase from soybean leaves, this enzyme was stable to prolonged dialysis in the absence of glutathione.

Inorganic phosphate was found to be essential for maximum enzymatic activity. The presence of 10^{-2}M potassium phosphate in assays of the enzyme which had been isolated and purified in tris(hydroxymethyl)aminomethane (Tris) buffer caused a 30 per cent. increase in activity. A similar dependence on inorganic phosphate has been demonstrated for the nitrate reductase of *Neurospora* by Kinsky and McElroy (1958).

Nitrate reductase from wheat embryos was inactivated by 38, 96, and 100 per cent. after 5 min heating to 30, 45, and 60°C respectively. Approximately 50 per cent. of the enzymatic activity was lost after storage for 1 week at −15°C.

(*f*) *Intracellular Distribution*

Embryos from germinating wheat were ground in 0·4M sucrose containing 0·05M potassium phosphate, pH 7·4, and 10^{-3}M glutathione. The grindate was centrifuged at low speed (approximately 100 *g*) to remove unbroken cells, and the supernatant solution was further centrifuged at 105,000 *g* in a Spinco ultracentrifuge for 30 min. All the nitrate reductase activity present in the supernatant solution from the low speed centrifugation was recovered in the supernatant solution following high speed centrifugation. The enzyme is thus located in the soluble, cytoplasmic fraction of the cell, rather than in the particulate fraction.

(*g*) *Identification of the Metal Component*

The extreme sensitivity of the enzyme to potassium cyanide and sodium azide (see Table 2) is strongly indicative of an essential heavy metal component. Nicholas and Nason (1954, 1955*a*) have demonstrated conclusively that molybdenum is an essential component of nitrate reductase from *Neurospora* and soybean leaves. They accomplished this by dialysis of the enzyme against potassium cyanide solution, subsequent removal of the metallocyanide complex and excess cyanide by dialysis against purified buffer, and then specific reactivation of the now inactive enzyme with molybdate salts. Repeated attempts to apply this technique to the DPNH-specific wheat nitrate reductase were unsuccessful. In every case, dialysis against purified buffer to remove excess cyanide and the metallocyanide complex resulted in restoration of activity to the enzyme, and no stimulation in activity could be obtained upon addition of a range of possible metal salts. The restored activity could still be inhibited by cyanide. This result indicates that either the metallocyanide complex is not dissociable from the enzyme, or that impurities in the purified buffer or the assay ingredients were sufficient to reactivate the enzyme fully. In an endeavour to eliminate the latter possibility, buffer salts (phosphate or Tris) were purified either by the copper sulphide co-precipitation method of Nicholas (1952), or by extraction with 8-hydroxyquinoline in chloroform at pH 4 and 7·5 (Gentry and Sherrington 1950). In several experiments the assay ingredients (DPNH, FAD, potassium nitrate) were also extracted with 8-hydroxyquinoline. The failure to obtain an inactive "metal-free" enzyme preparation under these conditions would suggest that the metallocyanide complex is not dissociable from the enzyme, although the possibility of trace metal impurities in the reagents has not been entirely eliminated.

Vanadate ion is a powerful inhibitor of the enzyme from young wheat embryos (Table 2). A final concentration of approximately 3×10^{-5}M sodium orthovanadate caused 50 per cent. inhibition. This suggested that the related molybdate ion might be the metal component of the enzyme. However, the inhibition by vanadate was not reversed by a 25-fold excess of sodium molybdate, indicating a non-competitive type of inhibition. Sodium tungstate at a final concentration of 10^{-3}M was without effect on enzymatic activity.

(*h*) *Other Enzymatic Activities*

The purest preparations of wheat embryo nitrate reductase contain an active DPNH–2,3′,6-trichlorophenolindophenol reductase and a DPNH–mammalian cytochrome *c* reductase. These activities are not affected by the addition of 5×10^{-4}M potassium cyanide. Kinsky and McElroy (1958) have demonstrated that TPNH–cytochrome *c* reductase is closely associated with the TPNH-specific nitrate reductase of *Neurospora*. Their evidence indicates that in *Neurospora* one enzyme system is involved in both nitrate and cytochrome *c* reduction, and they suggest that a flavin reductase catalyses the first step for both processes.

Table 3

COMPARISON OF THE PROPERTIES OF THE NITRATE REDUCTASES FROM SOYBEAN LEAVES AND FROM GERMINATING WHEAT

Property	Nitrate Reductase from Soybean Leaves*	Nitrate Reductase from Germinating Wheat
pH optimum	6·0	7·4
Pyridine nucleotide specificity	Non-specific	DPNH-specific
50 per cent. inhibition by potassium cyanide at	$2 \cdot 5 \times 10^{-4}$M	$1 \cdot 6 \times 10^{-6}$M
50 per cent. inhibition by sodium azide at	$1 \cdot 6 \times 10^{-4}$M	2×10^{-5}M
50 per cent. inhibition by *p*-chloromercuribenzoate at	10^{-6}M	10^{-5}M
Requirement for inorganic phosphate	Not known	Positive
Stability to dialysis in absence of glutathione	Unstable	Stable
Flavin specificity	FAD-specific	FAD-specific
Nature of the metal prosthetic group	Molybdenum	Not known
K_s for the DPNH–enzyme complex	3×10^{-5}M	8×10^{-6}M
K_s for the nitrate–enzyme complex	$7 \cdot 5 \times 10^{-3}$M	$3 \cdot 8 \times 10^{-4}$M

* Data taken from Evans and Nason (1953) and Nicholas and Nason (1955*a*); the values for 50 per cent. inhibition by potassium cyanide and sodium azide were obtained by interpolation of their data.

IV. DISCUSSION

The above experiments demonstrate the presence of a DPNH-specific nitrate reductase in embryos of germinating wheat. It is a soluble enzyme, which requires the addition of FAD for maximum activity. Potassium cyanide and sodium azide cause 50 per cent. inhibition of enzyme activity at $1 \cdot 6 \times 10^{-6}$M and 2×10^{-5}M respectively. This strongly suggests the presence of an essential metal component in the system. The enzyme from germinating wheat thus possesses the same general properties as the enzyme found in soybean leaves by Evans and Nason (1953), but the two enzymes differ markedly in some details. A comparison of some of the properties of the wheat embryo nitrate reductase with those already published for the nitrate reductase of soybean leaves (Evans and Nason 1953; Nicholas and Nason 1955*a*) is given in Table 3. The principal differences are in pH optimum, pyridine nucleotide specificity, and sensitivity to some inhibitors, especially cyanide. The enzyme from wheat has a significantly greater affinity for both the substrates nitrate and DPNH.

Perhaps the most interesting difference physiologically is that of pyridine nucleotide specificity. The metalloflavoprotein nitrate reductases which have been described in various organisms show a range of pyridine nucleotide specificities. The nitrate reductase from *Escherichia coli* (Nicholas and Nason 1955*b*), from the bacteroids of *Rhizobium japonicum* (Cheniae and Evans 1956), and that from wheat embryos are specifically linked to DPNH. The enzyme from *Neurospora* (Nason and Evans 1954) is TPNH-specific, while that from soybean leaves (Evans and Nason 1953) can be linked to either DPNH or TPNH with equal effectiveness.

The possible physiological significance of the DPNH-specific nitrate reductase will be the subject of future experiments. Preliminary results indicate that in higher plants DPNH-specificity may be a characteristic of the nitrate reductase of embryos—in both the pregerminal and early postgerminal stages. The induced nitrate reductase of pregerminal developing wheat embryos described by Rijven (1958) has since been found to be DPNH-specific. Furthermore, we have found that germinating soybean seeds appear to contain only a DPNH-specific nitrate reductase, whilst older tissues of both soybean (Evans and Nason 1953) and wheat (Spencer and Rijven, unpublished data) contain the non-specific enzyme. Consistent with this theory is the recent finding of Egami *et al.* (1957) that macerates of cotyledons of 2-day-old *Vigna sesquipedalis* catalyse the reduction of nitrate to nitrite by DPNH but not by TPNH.

V. Acknowledgment

The work reported in this paper arose out of collaborative experiments with Dr. A. H. G. C. Rijven, Division of Plant Industry, C.S.I.R.O., to whom the author is grateful for his interest and valuable discussions.

VI. References

Cheniae, G., and Evans, H. J. (1956).—"Inorganic Nitrogen Metabolism." p. 184. (Ed. W. D. McElroy and B. Glass.) (The Johns Hopkins Press: Baltimore.)

Egami, F., Ohmachi, K., Iida, K., and Tanaguchi, S. (1957).—*Biochimia* **22**: 122–34.

Evans, H. J., and Nason, A. (1953).—*Plant Physiol.* **28**: 233–54.

Gentry, C. H. R., and Sherrington, L. G. (1950).—*Analyst* **75**: 17–21.

Kinsky, S. C., and McElroy, W. D. (1958).—*Arch. Biochem. Biophys.* **73**: 466–83.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951).—*J. Biol. Chem.* **193**: 265–75.

Medina, A., and Nicholas, D. J. D. (1957).—*Biochim. Biophys. Acta* **23**: 440–2.

NASON, A., and EVANS, H. J. (1954).—*J. Biol. Chem.* **202**: 655–73.

NICHOLAS, D. J. D. (1952).—*Analyst* **77**: 629–42.

NICHOLAS, D. J. D. (1958).—*J. Gen. Microbiol.* **18**(1): iv.

NICHOLAS, D. J. D., and NASON, A. (1954).—*J. Biol. Chem.* **207**: 353–60.

NICHOLAS, D. J. D., and NASON, A. (1955*a*).—*Plant Physiol.* **30**: 135–8.

NICHOLAS, D. J. D., and NASON, A. (1955*b*).—*J. Bact.* **69**: 580–3.

OCHOA, S. (1955).—In "Methods in Enzymology". Vol. 1. p. 702. (Ed. S. P. Colowick and N. O. Kaplan.) (Academic Press Inc.: New York.)

RAFTER, G. W., and COLOWICK, S. P. (1957).—In "Methods in Enzymology". Vol. 3. p. 887. (Ed. S. P. Colowick and N. O. Kaplan.) (Academic Press Inc.: New York.)

RIJVEN, A. H. G. C. (1958).—*Aust. J. Biol. Sci.* **11**: 142–54.

FORMATION OF A POLYSACCHARIDE IN SUGAR-CANE

By R. I. Nicholson* and B. Lilienthal†

[*Manuscript received October* 27, 1958]

Summary

The isolation of a polysaccharide from harvested, stored sugar-cane is described. The polysaccharide is characterized by a low content of 1,6-linkages (29 per cent. approx.) and a relatively high content of 1,4-linkages (60 per cent. approx.) which values are unusual for a dextran. It is susceptible to attack by certain amylases, yielding oligosaccharides. This type of polysaccharide has been isolated only from sugar-cane and the *Leuconostoc* associated with it yields normal dextran when cultured under a variety of conditions.

I. Introduction

Dextran, formed by the action of dextransucrase on sucrose, has long been recognized as a contaminant of juices from sugar-cane (Walton and Fort 1931; McCalip and Hall 1938). According to work in this Department, dextrans occurring in juices can be derived from two sources: (i) normally, from the juice undergoing dextranous fermentation after expression from the cane; (ii) from intact cane which forms a polysaccharide of low percentage 1,6-linkages during a period of delay before it is crushed, and which is located first in the nodal zones of the stalk and later in the internodal zones in small amounts.

Since the occurrence of the latter type of polysaccharide in cane is usually noted late in the harvesting season, after rain, and is associated with a delay between cutting and crushing, the following postulate was advanced by the authors for its formation. The cane stalk is frequently contaminated with *Leuconostoc* and also, after burning, is splashed with juice from bursting stalks. This infected material is washed down the stalk by rain and becomes lodged behind the point of attachment of the leaf and is in contact with the bud. The buds are corky at this time of the season and have been shown to be permeable to *L. mesenteroides*. It was thus considered that polymeric material formed in the nodal region by this mechanism of infection through the bud.

This report is concerned with some aspects of the mechanism of formation of this polysaccharide, its isolation, properties, and characterization. It has been found that the mechanism of its formation is more complex than previously supposed and cannot be explained solely in terms of the activity of *L. mesenteroides*.

---

*Research Department, Colonial Sugar Refining Co. Ltd., Sydney.

†Research Department, Colonial Sugar Refining Co. Ltd., Sydney; present address: Department of Bacteriology, University of Melbourne.

II. Materials

(i) *Cane*.—Commercial sugar-cane of various varieties was obtained from Queensland and New South Wales.

(ii) *Organisms*.—Type cultures of *L. mesenteroides* were employed on occasions but more frequently strains of this organism were isolated from sugar-cane, using the medium of McCleskey, Faville, and Barnett (1947).

(iii) *Reagents and Enzymes*.—Chemically pure reagents were used for the periodate oxidations in linkage determination. The amylases used were filtered saliva, "Bacterase" (Norman Evans and Rais Ltd., Stockport, England), or filtered fresh cane juice which contains a natural amylase.

III. Methods

(*a*) *Inoculation of Cane Stalks*

Pieces of cane approx. 3 in. long were cut at the nodal and internodal regions of the stalk. Holes were cut with a stainless steel cork borer for inoculation with *L. mesenteroides*. The plugs were replaced and the sections of cane immersed in cane juice and autoclaved at 15 lb pressure for 30 min. Using an aseptic technique, the plugs were removed, a known volume of bacterial inoculum was deposited in the cane, and the plug replaced. After a variable period of incubation at 28°C in sterilized jars, the sections were analysed for dextrans and counts made of the numbers of *Leuconostoc* present.

(*b*) *Infection of Cane through the Buds*

Green stalks of cane with undamaged buds were obtained and alternate buds were walled off by a dam of paraffin wax leaving the bud exposed. A small volume (0·1 ml) of a culture of *L. mesenteroides* was placed on the bud and after a suitable time the interior of the stalk was examined for this organism.

(*c*) *Isolation of* Leuconostoc *from Cane*

Sections of cane approx. 1 in. long were cut from the nodal or internodal regions of the stalk. The rind was cut off and the sample cut into small sections longitudinally. After weighing, the sections were disintegrated by high-speed stirring in a known volume of sterile water. Fibrous material was removed on a Buchner funnel and an aliquot of the filtrate was placed in a sterile tube for subsequent counting on the medium of McCleskey, Faville, and Barnett (1947). Polysaccharide was determined in the remaining filtrate after acidification with a 10 per cent. solution of trichloroacetic acid, either by direct weighing after precipitation with 50 per cent. v/v ethanol (Whiteside-Carlson and Carlson 1949), or by measurement of "colloidal haze" (Nicholson and Horsley, unpublished data).

Water blanks were routinely carried out and failed to show significant contamination or carry-over of *Leuconostoc*. After plating out, the plates were incubated at 30°C overnight.

TABLE 1

PROPERTIES OF THE POLYSACCHARIDE FROM SUGAR-CANE AND OF BACTERIAL ORIGIN

(*a*) Polysaccharide with high 1,4-linkage content from sugar-cane samples

Sugar-cane Sample No.	Media	Specific Rotation (degrees)	Periodate Consumed (moles)	Formic Acid Liberated (mole)	Calculated Linkage (%)		
					1,6-	1,4-	1,3-
1	Top nodes	202·1	1·31	0·29	29	73	−2
	Bottom nodes	199·6	1·31	0·29	29	73	−2
	Mixed nodes	192·8	1·27	0·34	34	59	7
2	Inoculated nodes	—	1·17	0·27	27	63	10
	Uninoculated nodes	—	0·65	0·19	19	27	54
3	Stored cane	196·9	1·26	0·28	28	70	2
4	Inoculated nodes	198·0	1·16	0·35	35	46	19

TABLE 1 (*Continued*)

(*b*) Polysaccharide with high 1,6-linkage content formed by *L. mesenteroides* when grown on synthetic media and in various sugar-cane juices

L. mesenteroides Strain No.	Media	Specific Rotation (degrees)	Periodate Consumed (moles)	Formic Acid Liberated (mole)	Calculated Linkage (%)		
					1,6-	1,4-	1,3-
1*	5% sucrose	219·0	1·79	0·85	85	9	6
	20% sucrose	183·5	1·67	0·83	83	1	16
	20% sucrose						
	Stored 10 days	211·6	1·66	0·84	84	—2	18
	,, 17 ,,	199·3	1·83	0·90	90	3	7
	,, 42 ,,	216·0	1·76	0·81	81	14	5
1	Sugar-cane juice	192·9	1·91	0·94	94	3	3
	,, ,, ,,	204·7	1·86	0·96	96	—6	10
	,, ,, ,,	203·4	1·92	0·90	90	12	—2
	,, ,, ,,	200·8	1·86	0·89	89	7	4
	,, ,, ,,	200·2	1·90	0·96	96	—2	6
	,, ,, ,,	196·9	1·93	0·87	87	19	—6
	,, ,, ,,	199·7	2·04	0·97	97	10	—7
	,, ,, ,,	200·3	1·87	0·88	88	11	1
	,, ,, ,,	203·1	1·80	0·87	87	6	7
2	,, ,, ,,	199·5	1·86	0·96	96	—6	10
3	,, ,, ,,	191·1	1·44	0·68	68	8	24
	,, ,, ,,	190·6	1·85	0·93	93	—1	8
4	,, ,, ,,	187·7	1·74	0·87	87	0	13
5†	,, ,, ,,	201·1	1·92	0·94	94	4	2

*Strains 1–4 were isolated from sugar-cane samples 1–4. †Strain 5 was ATCC 8357.

(*d*) *Isolation of the Polysaccharides from Cane*

Juice was expressed from cane using either a three-roll mill or a small hydraulic press. A 10 per cent. solution of trichloroacetic acid (0·2 vol.) was added to the juice which was then filtered employing 2 per cent. of a filter aid such as "Super-Cel". Ethanol (1 vol.) was added with slow stirring and the precipitated polysaccharide collected, drained free from ethanol, and redissolved in water employing high-speed stirring. The process of solution and precipitation was repeated twice more with a filtration of the solution (filter aid) interposed. Ethanol was removed during vacuum concentration. Soluble solids were determined by drying and a small correction for ash applied. The solutions were stored under refrigeration.

Table 2

POLYSACCHARIDE FORMATION IN SUGAR-CANE SECTIONS INOCULATED WITH L. MESENTEROIDES

Results are from two samples of each type of section

Time (days)	Node		Internode	
	No. of *L. mesenteroides* Cells ($\times 10^8$)	Polysaccharide (mg/g dry wt.)	No. of *L. mesenteroides* Cells ($\times 10^8$)	Polysaccharide (mg/g dry wt.)
1	3·0	26·4	10·0	Nil
	8·0	Nil	3·5	1·8
2	20·0	19·6	4·5	2·3
	2·5	16·0	2·5	Nil
3	3·0	144·0	8·5	Nil
	5·5	8·2	7·5	Nil
4	15·0	Nil	100·0	Nil
	7·5	Nil	25·0	Nil

The specific rotation of the polysaccharide was determined on part of the sample. It was usually necessary to apply a very mild hydrolysis (3 per cent. acetic acid, held 1 hr at 70°C) to improve the clarity before readings on the polarimeter could be made. Treatment with dilute potassium hydroxide or formamide did not remove the colloidal haze. Enzymatic and chromatographic studies were based on conventional techniques.

(*e*) *Periodate Oxidation of the Polysaccharide*

Information on the structure of the polysaccharide was sought by periodate oxidation. The method employed was essentially that of Hehre (1951) with slight modifications. Potassium periodate was used instead of the sodium salt and the concentration of some of the solutions was altered to permit more accurate pipetting.

The polysaccharide was treated with 2·5–3·5 moles potassium periodate per mole of anhydroglucose and, after tests on dextrans and starches, a storage period of 72 hr in the dark before analysis was adopted.

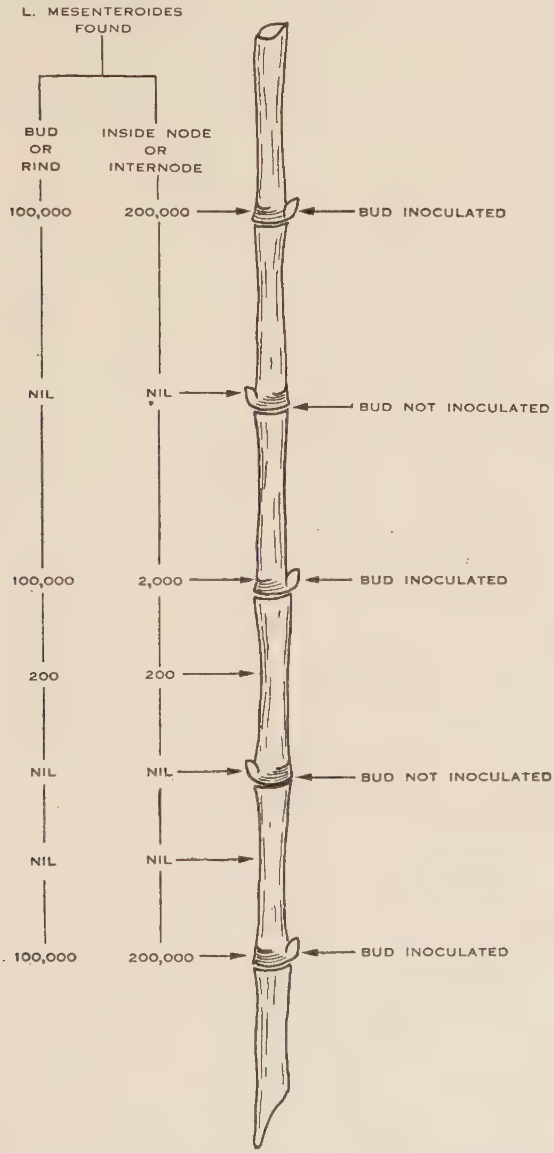


Fig. 1.—Inoculation of alternate sugar-cane buds with *L. mesenteroides* and subsequent bacterial counts after 24 hr.

Table 1 lists the periodate consumption and formic acid liberation as moles per mole of anhydroglucose. The calculations for the linkage types were made from these values according to the methods of Jeanes and Wilham (1950), Rankin and Jeanes (1954), and Sloan *et al.* (1954).

IV. Results

(a) *Inoculation of Cane Stalks*

Inoculation of sterilized cane with suspensions of *L. mesenteroides* showed that, although the bacteria remained alive inside the cane, the amount of the polysaccharide formed was variable and too small in quantity for analysis. The numbers of viable organisms were not related to the amount formed (Table 2). Accordingly the attempted formation of the polysaccharide within the cane by this method was discontinued.

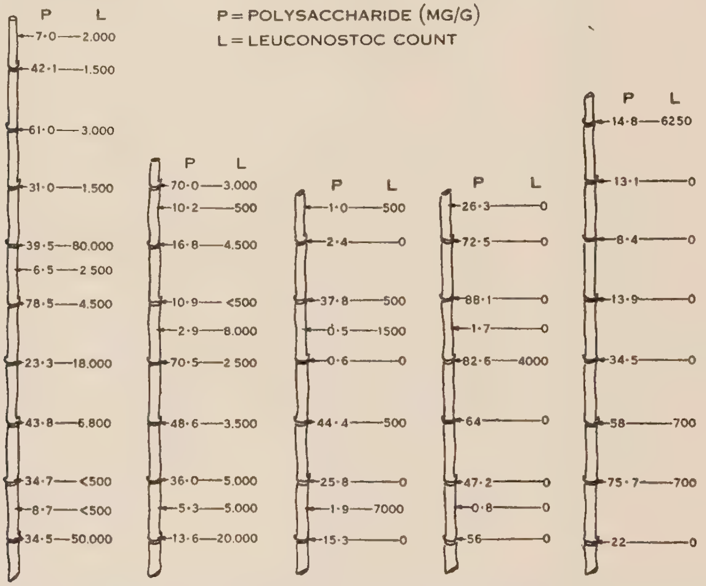


Fig. 2.—Spontaneous formation of the polysaccharide in sugar-cane. *Leuconostoc* counts for the same regions of the cane are also indicated.

(b) *Infection of Cane through the Buds*

Inoculated buds were found to have over 100,000 *Leuconostoc* cells, whereas none were found in the uninoculated buds. The interior tissue of the nodes under the inoculated buds contained organisms whereas none was found under uninoculated buds. Figure 1 illustrates the results obtained. It should be noted that the polysaccharide was not formed in this stalk of cane.

Repetition of the experiment on more mature cane later in the season showed a similar penetration of the buds by the bacteria. However, in this experiment, polysaccharide was found in the nodes and internodes, but the amount observed was unrelated to the numbers of *Leuconostoc*. The greatest amount found (690 mg) was present in an uninoculated node in which no *Leuconostoc* cells were detected.

(c) *Spontaneous Formation of the Polysaccharide in Cane*

A further sample of cane was found to contain both the polysaccharide and *Leuconostoc* (Fig. 2). The number of organisms in various regions of five stalks of cane was not related to the quantity of the polysaccharide found in the corresponding regions of the stalks. Since this batch of cane was examined several weeks after harvesting, the organisms may have been no longer viable and information on this aspect was sought. Figure 3 shows cane initially free of *Leuconostoc* which increased in bacterial count over a period of 14 days yet showed no formation of the polysaccharide.

(*d*) *Characterization of the Polysaccharide Formed in Cane*

The material precipitated by a 50 per cent. concentration of ethanol and purified by two further similar precipitations was different in constitution from normal dextran. Table 1 shows that the specific rotation is similar to that of normal dextran but the polysaccharide contains a much smaller proportion of 1,6-linkages and a higher proportion of 1,4-linkages than normal dextran. The strains of *L. mesenteroides* isolated from the polysaccharide-containing material when cultured in cane juice or synthetic media under a variety of conditions yielded only dextrans of normal constitution.

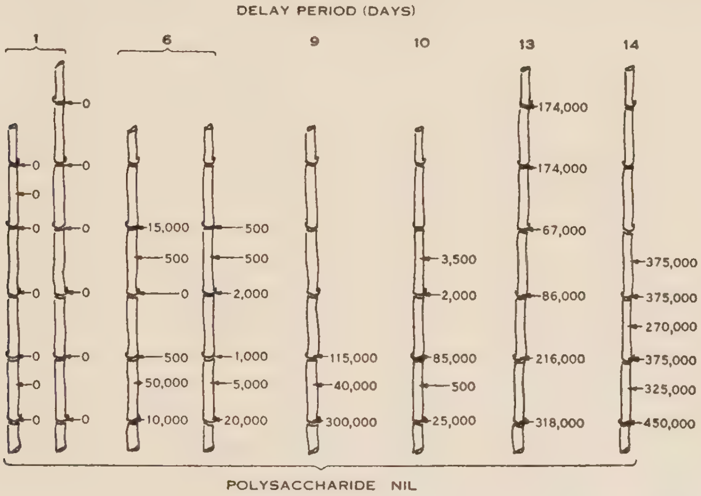


Fig. 3.—Increase in *Leuconostoc* counts in sugar-cane over a period of 14 days without formation of the polysaccharide.

(*e*) *Action of Enzymes on the Polysaccharide Isolated from Cane*

The polysaccharide was attacked by certain amylases as shown in Table 3. Salivary amylase sharply differentiated between normal dextran and this material. Cane-juice amylase (Nicholson and Horsley 1958) partially degraded the polysaccharide but this enzyme is in low concentration and it is not known if its action would continue on further incubation.

The polysaccharide gave two fractions (A and B) after treatment with salivary amylase followed by ethanol fractionation. The polysaccharide (20 ml, 4·75 per cent. solids) was incubated for 5 hr at 40°C with 2 ml phosphate buffer (pH 6·9)

TABLE 3

ACTION OF ENZYMES AT 40°C ON THE POLYSACCHARIDE ISOLATED FROM SUGAR-CANE AND ON POLYSACCHARIDES OF BACTERIAL ORIGIN

Origin of Polysaccharide	Isolated from:	Enzyme	Treatment		Polysaccharide in Aliquot (mg)		Removal (%)
			Concn.	Time (min)	Before	After	
Sugar-cane*	Top nodes	Salivary amylase	1 ml/25 ml	80	14·80	0	100
	Mixed nodes	,, ,,	,, ,,	80	13·80	0	100
	Whole cane	,, ,,	,, ,,	80	14·20	0	100
	Top nodes	Cane-juice amylase	5 ml/25 ml	60	11·60	10·25	11·7
	Mixed nodes	,, ,,	,, ,,	60	11·00	10·00	9·1
	Whole cane	,, ,,	,, ,,	60	12·50	11·50	8·0
Bacterial†	Sugar-cane juice	Salivary amylase	1 ml/25 ml	60	9·50	9·25	2·6
	Synthetic medium	,, ,,	,, ,,	60	11·20	10·75	4·0
	Synthetic medium +20% sucrose	,, ,,	,, ,,	60	10·50	10·25	2·4

*Only one sample of sugar-cane used.

†*L. mesenteroides* isolated from whole sugar-cane used above and grown on the cane juice from the same cane. *L. mesenteroides* was also grown on the other synthetic media listed.

and 4 ml saliva. Fraction A was precipitated in 68 per cent. ethanol after filtering off and discarding a faint precipitate which formed at 50 per cent. alcohol concentration. Fraction B was obtained by evaporation to dryness of the filtrate from 80 per cent. ethanol. Some properties of these fractions are given in Table 4.

TABLE 4

PROPERTIES OF FRACTIONS OBTAINED BY THE ACTION OF SALIVARY AMYLASE ON THE POLYSACCHARIDE ISOLATED FROM SUGAR-CANE FOLLOWED BY ETHANOL FRACTIONATION

Property	Original Polysaccharide	Fraction A	Fraction B
Weight	0·950 g	0·443 g	0·370 g
Degree of polymerization	377	22	6
1,6-linkages (%)	28	43	70
1,4-linkages (%)	70	54	7

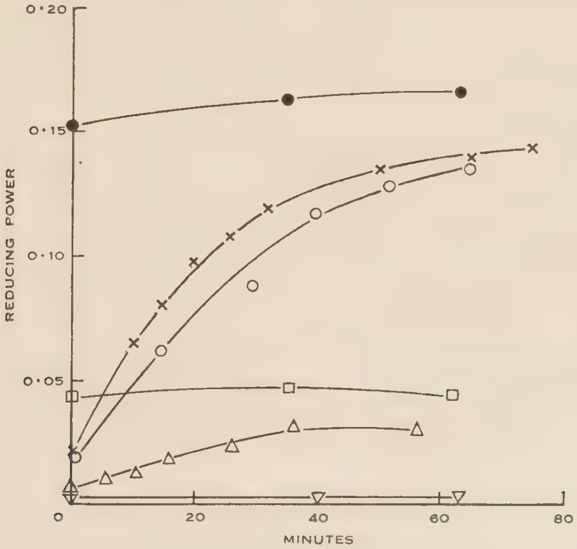


Fig. 4.—Action of salivary amylase (△) and "Bacterase" (▽) on the polysaccharide and of salivary amylase on fractions A (□) and B (●) of this polysaccharide. The action of salivary amylase (○) and "Bacterase" (×) on soluble starch is shown for comparison. Reducing power is expressed as: moles reducing sugar/moles glucose anhydride.

The values here given for fraction B may include traces of reducing sugars which would not be removed by the method of preparation.

The action of salivary amylase and of "Bacterase" on the polysaccharide and of salivary amylase on fractions A and B is illustrated in Figure 4. The fractions are included to show that they are "limit" products. For comparison, the action of these two amylases on soluble starch is also shown. The polysaccharide has a high proportion of 1,4-linkages (Table 4) and is attacked by salivary amylase, though not producing a large increase in reducing power. Rather, lower polymers are produced.

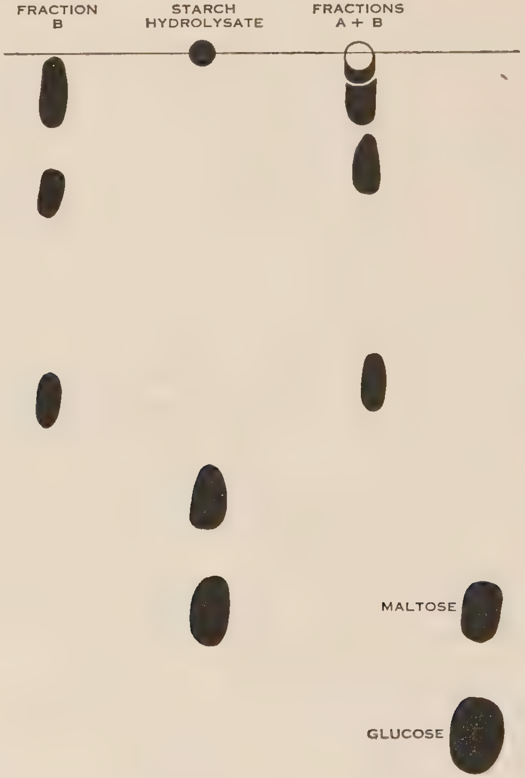


Fig. 5.—Paper chromatography of fraction B and of fractions A and B together compared with that of starch hydrolysate. The solvent used was n-butanol–dimethyl formamide–water (2 : 1 : 1 v/v). Maltose and glucose ($R_F = 0 \cdot 52$) have been run on the same chromatogram as markers.

(*f*) *Paper Chromatography of the Polysaccharide*

Preliminary paper chromatography work with fractions A and B and with fraction B alone has been carried out and some of the results obtained are illustrated in Figure 5. The n-butanol–dimethyl formamide–water (2 : 1 : 1 v/v) solvent of Cramer and Steinle (1955) was used since it gave better resolution of oligosaccharides than n-butanol–acetic acid–water. The pattern of spots for the fractionated polysaccharide is quite distinct from that of starch hydrolysate (using salivary amylase) which was run on the same chromatogram for comparison. Reducing sugar is not detectable on the chromatogram.

V. Discussion

Sugar-cane forms, under certain conditions, a polysaccharide which, at first, was thought to be a modified dextran. The constitution of the material encountered is outside the range of dextrans found in the survey of 96 varieties from various sources by Jeanes *et al.* (1954). The new polysaccharide is not a mixture of starch and dextran since it yields no colour with iodine, is not attacked by "Bacterase", and yields a distinct pattern by paper chromatography.

Whenever this polysaccharide forms it does so under conditions which have been favourable to the growth of *Leuconostoc* in the nodes although the organism, at the time of examination, may have been non-viable. The composition of the polysaccharide suggests that it may be formed either by the priming action of dextran from dextransucrase upon another polysaccharide-synthesizing system or by plant enzymes alone.

VI. Acknowledgments

The work described in this paper was carried out in the Research Department of the Colonial Sugar Refining Co. Ltd., Sydney. The authors wish to thank the Company for permission to publish this paper and Miss M. Horsley and Miss G. McIntyre for technical assistance.

VII. References

Cramer, F. D., and Steinle, D. (1955).—*Liebigs Ann.* **595**: 81.

Hehre, E. J. (1951).—*J. Biol. Chem.* **192**: 161.

Jeanes, A., and Wilham, C. A. (1950).—*J. Amer. Chem. Soc.* **72**: 2655.

Jeanes, A., et al. (1954).—*J. Amer. Chem. Soc.* **76**: 5041.

McCalip, M. A., and Hall, H. H. (1938).—Proc. 6th Congress I.S.S.C.T.

McCleskey, C. S., Faville, L. W., and Barnett, R. O. (1947).—*J. Bact.* **54**: 697.

Nicholson, R. I., and Horsley, M. (1958).—*Int. Sugar J.* **60**: 260.

Rankin, J. C., and Jeanes, A. (1954).—*J. Amer. Chem. Soc.* **76**: 4435.

Sloan, J. W., et al. (1954).—*J. Amer. Chem. Soc.* **76**: 4429.

Walton, C. F. Jr., and Fort, C. A. (1931).—*Ind. Engng. Chem.* **23**: 1295.

Whiteside-Carlson, V., and Carlson, W. W. (1949).—*J. Bact.* **58**: 135.

THE ADSORPTION BY *AEROBACTER AEROGENES* OF *p*-HYDROXYBENZOIC ACID AND ITS ESTERS AND OF SODIUM BENZOATE AT DIFFERENT pH LEVELS

By R. G. H. Barbour*

[*Manuscript received November* 17, 1958]

Summary

Heavy suspensions of *Aerobacter aerogenes* were used to measure the adsorption of sodium benzoate and of *p*-hydroxybenzoic acid and its methyl, ethyl, propyl, and butyl esters. It was found that the adsorbability of the esters increased logarithmically with increasing chain length of the ester group. Surface activity also increased logarithmically with chain length. A close parallel was observed between the adsorption of the compounds and their antimicrobial effectiveness found by earlier workers.

The adsorption of sodium benzoate increased as the acidity of the suspending medium increased. The ratio of the coefficients of adsorption of the unionized molecules to the anions was 5·7. The increase in adsorbability was parallelled by an increase in surface activity.

I. Introduction

The correlation which has been found in homologous series of antimicrobial compounds between the number of carbon atoms in the chain and the logarithms of such properties as toxic concentration, water solubility, surface activity, vapour pressure, and partition coefficient between oil and water suggests that the toxicity of these compounds may well be related to their adsorbability (Ferguson 1939). This paper reports the measurement of the adsorption by *Aerobacter aerogenes* of *p*-hydroxybenzoic acid and some of its esters. The antimicrobial activity of these compounds has been investigated earlier (Sabalitschka 1931; Murrell and Vincent 1950).

Trim and Alexander (1949) suggested that inhibitory compounds which ionize to give an organic anion should have less tendency to become adsorbed in the ionized form. This paper also reports the results of experiments carried out to determine the relative adsorbability of the ionized and unionized forms of sodium benzoate.

II. Material and Methods

The bacterium used was *A. aerogenes* strain 32 and was obtained from Professor J. M. Vincent, School of Agriculture, University of Sydney. The same organism was used by Murrell and Vincent (1950) and by Barbour and Vincent (1950).

The organic compounds employed were as follows: sodium benzoate (B.D.H. Ltd.); *p*-hydroxybenzoic acid, stock sample purified by recrystallization from

---

* School of Biological Sciences, University of New South Wales, Ultimo, Sydney.

distilled water; methyl, ethyl, propyl, and butyl *p*-hydroxybenzoates, prepared by Dr. G. W. K. Cavill of the University of New South Wales. All estimates of concentrations of the organic compounds were made by using ultraviolet light absorption. The instrument employed was a Unicam quartz spectrophotometer, model SP 500.

The buffers used were either M/15 Na_2HPO_4–M/15 KH_2PO_4 for pH 5·2 and above or M/10 citric acid–M/5 Na_2HPO_4 for pH levels below 5·2.

Surface tension measurements were made using a du Noüy tensiometer with a platinum ring.

III. PROCEDURE

(*a*) *Bacterial Concentration*

The bacterial cells used were grown on bottle slopes of veal agar for 24 hr and then harvested and suspended in quarter-strength Ringer's solution. The cells were washed twice with Ringer's solution and were filtered through a thin layer of cotton wool after the first washing to remove any suspended agar. After the second washing the number of bacteria per ml was estimated optically from a sample suitably diluted in Ringer's solution.

(*b*) *Adsorption of* p-*Hydroxybenzoic Acid and its Esters*

From the estimate of cell numbers, the volume of cell suspension required to give a concentration of $6\cdot4\times10^6$ cells per ml in contact with the compound was calculated by simple proportion. The calculated volume of cell suspension was pipetted into each of four centrifuge tubes and centrifuged in a high-speed refrigerated centrifuge at approximately 12,000 r.p.m. for 15 min. The supernatant was removed and to the deposited cells in each of three centrifuge tubes 4 ml of compound in buffer at pH 7·0 were added. Buffer solution only was added to the fourth tube of cells which was used subsequently as an optical control. Cells and suspending medium in each of the tubes were thoroughly mixed by means of Pasteur pipettes, allowed to stand for 10 min, and then centrifuged as previously. The supernatants were removed and stored in the refrigerator for estimation of the concentration of the compound on the following day.

Before measurement the supernatants were suitably diluted and the ultraviolet absorption compared with the supernatant of the optical control (similarly diluted) which was used to allow for any ultraviolet-absorbing compounds released from the cells. The readings obtained were compared with a standard curve prepared from the stock solutions of the compound. The amount of the compound adsorbed onto the cells was found by difference.

To determine whether the adsorption of the compound was causing the release of ultraviolet-absorbing compounds from the cells the following method was used: the supernatant from cells plus the compound was taken, and after suitable dilution, measurements of optical density were made over a series of wavelengths in the ultraviolet range. The supernatant, similarly diluted, from cells plus buffer solution only was used as the optical control. A series of readings was made on the original stock solution (diluted identically with the supernatants) over the same series of wavelengths as the supernatants. The release of ultraviolet-absorbing compounds could be detected by comparison of the ratio of the optical density of the supernatant to the optical density of the stock solution at the various wavelengths. A relatively constant ratio indicated that little or no ultraviolet-absorbing compounds were being released from the cells. Conversely, a variable ratio indicated the release of ultraviolet-absorbing compounds.

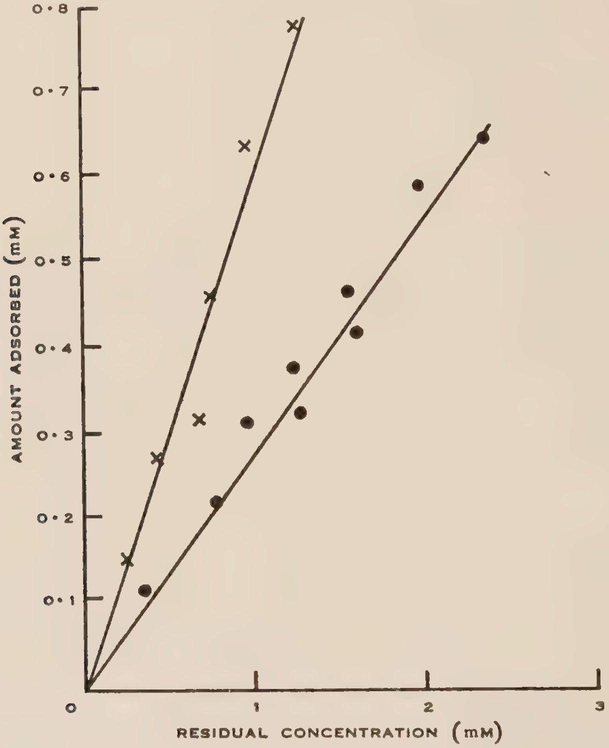


Fig. 1.—Adsorption of methyl (●) and ethyl (×) *p*-hydroxybenzoates.

All adsorption experiments carried out with *p*-hydroxybenzoic acid and its esters were at pH 7·0 and all experiments were at room temperature.

(*c*) *Adsorption of Sodium Benzoate at Different pH Levels*

The adsorption procedure was the same as that described for *p*-hydroxybenzoic acid and its esters except that the experiments were carried out at four different pH levels. Sodium benzoate was used in preference to the acid because of its greater solubility.

IV. Results

(*a*) *General*

Previous work (Barbour and Vincent 1950) indicated that an equilibrium between bacteria and inhibitor was established almost immediately after contact.

Preliminary adsorption experiments supported this view and showed that there was no difference in the amount of compound adsorbed at times of contact of 10 and 20 min. For this reason an adsorption time of 10 min has been used throughout this work. Preliminary experiments also established that the amount of compound adsorbed was small unless very heavy suspensions of bacteria were used.

TABLE 1

ADSORPTION OF *p*-HYDROXYBENZOIC ACID AND ITS ESTERS

Compound	A/R	Range of Concentrations Tested (mM)
p-Hydroxybenzoic acid	0·102	5 –15
Methyl ester	0·281	0·5– 3
Ethyl ester	0·593	0·4– 2
Propyl ester	1·137	0·3– 1
Butyl ester	2·750	0·1– 0·5

In all cases examined it was found that the relation between the amount of compound adsorbed and the residual concentration was linear. Comparison of the adsorbability of compounds has been made on the basis of the value of the ratio of the amount adsorbed (A) to the residual concentration (R). This value (A/R) represents the slope of the line fitted to the experimental points.

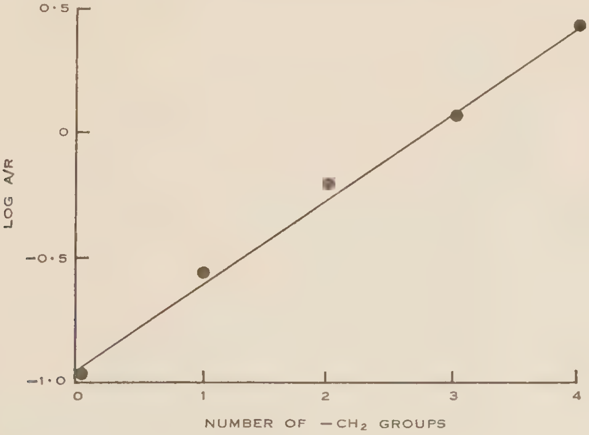


Fig. 2.—Effect of chain length of the ester group on the adsorption of *p*-hydroxybenzoic acid and its esters.

(*b*) *Adsorption of* p-*Hydroxybenzoic Acid and its Esters*

Typical adsorption results are shown in Figure 1 and the values of A/R for the series are given in Table 1. The logarithms of the A/R values plotted *v.* chain length of the ester group are shown in Figure 2.

Measurements were carried out to determine whether the adsorption of the compound caused the release of ultraviolet-absorbing compounds from the cells. Over the range of wavelengths from 240–280 mμ the ratio of the optical density of the supernatant to the optical density of the stock solution was relatively constant. Experiments carried out with ethyl *p*-hydroxybenzoate at concentrations of 1 and 2 mM at pH 3·0 gave similar results.

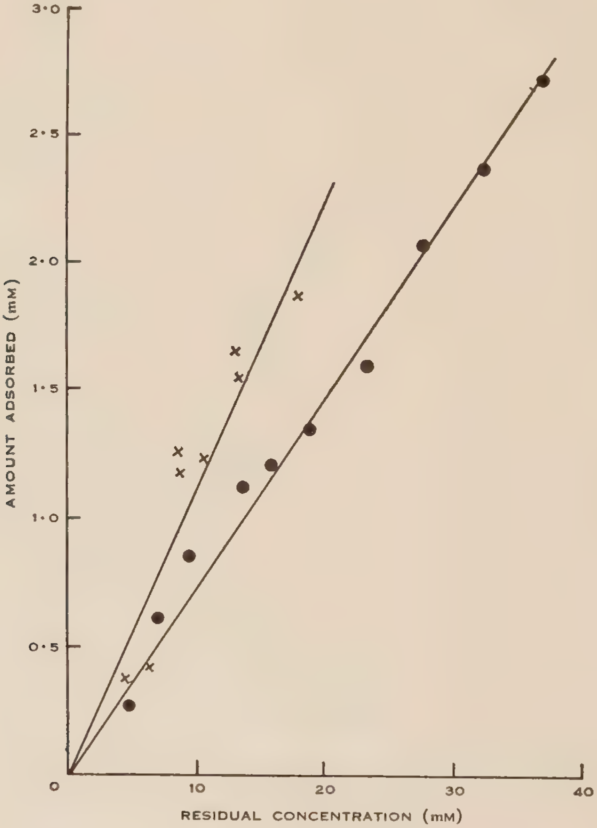


Fig. 3.—Adsorption of sodium benzoate at pH 7·1 (●) and pH 5·2 (×).

Surface tension measurements were made of 1 mM solutions of the compounds in water. The results are shown in Table 2. The logarithms of the surface tension reduction caused by the methyl, ethyl, and propyl esters when plotted against chain length of the ester group form a straight line but the value obtained for the butyl ester is less than expected, most probably owing to the relative insolubility of this compound.

(*c*) *Adsorption of Sodium Benzoate at Different pH Levels*

Two typical adsorption curves are shown in Figure 3. Comparison of the adsorption at the different pH levels is made in Table 3. In this table values of

A/R, A/I (ratio of amount adsorbed to initial concentration), percentage of unionized molecules, and range of concentrations used are given.

Surface tension measurements were made on solutions of benzoic acid in buffer at different pH levels. The acid was used because it was found impossible to obtain stable readings with the sodium salt. Antonoff (1945) reported similar difficulty when measuring the interfacial tension between water and phenol. Even with benzoic acid considerable time was necessary for the solution to reach stability. The results of these experiments are shown in Figure 4 and Table 4.

TABLE 2

REDUCTION OF SURFACE TENSION OF WATER BY 1 mM SOLUTIONS OF *p*-HYDROXYBENZOIC ACID AND ITS ESTERS

Compound	Surface Tension Reduction (dyn/cm)	Compound	Surface Tension Reduction (dyn/cm)
p-Hydroxybenzoic acid	0·26	Propyl ester	9·75
Methyl ester	0·45	Butyl ester	21·35
Ethyl ester	2·30		

TABLE 3

ADSORPTION OF SODIUM BENZOATE AT DIFFERENT pH LEVELS

pH	A/R	A/I	Per Cent Unionized	Range of Concentrations Tested (mM)
7·10	0·074	0·069	0·13	5–40
5·20	0·111	0·100	9·09	5–20
4·85	0·226	0·180	18·29	2–10
4·00	0·350	0·259	61·30	3–10

Experiments carried out to determine whether ultraviolet-absorbing compounds were being released from the cells by the adsorption of sodium benzoate indicated that at pH 3·0 the ratios of the optical densities were not constant but were relatively constant at pH 4·0 and above.

V. DISCUSSION

Trim and Alexander (1949) pointed out that the first step in biological systems is an uptake of the compound by adsorption (solution) in the extreme boundary layer or by adsorption upon the bounding surface. With non-surface-active substances such as chloroform they consider it more accurate to speak of adsorption in boundary layers rather than adsorption on the surface. With ionized substances and with surface-active unionized molecules the first step will be absorption. As the compounds investigated in this paper are of the latter type the term adsorption has been used.

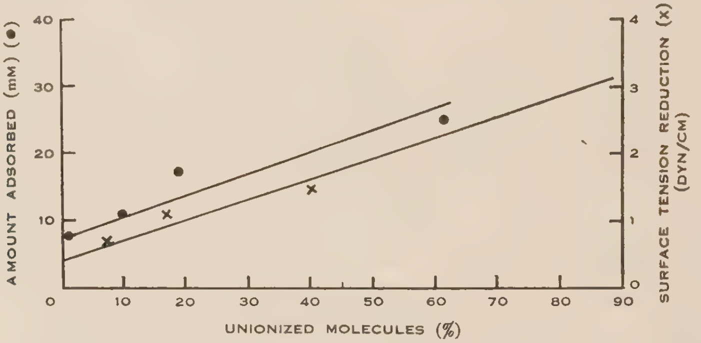


Fig. 4.—Relation of degree of dissociation to adsorption (●) and to surface activity (×).

(a) *Adsorption of* p-*Hydroxybenzoic Acid and its Esters*

The work with *p*-hydroxybenzoic acid and its esters has shown that the adsorption of these compounds at the concentrations used does not cause the release of ultraviolet-adsorbing compounds from the cells. This is apparent from the relatively constant ratios of the optical densities which was typical of the whole series.

Table 4

REDUCTION OF SURFACE TENSION BY 10 mM SOLUTIONS OF BENZOIC ACID IN BUFFER AT DIFFERENT pH LEVELS

pH	Surface Tension Reduction (dyn/cm)	Per Cent Unionized	pH	Surface Tension Reduction (dyn/cm)	Per Cent. Unionized
7·00	Not detectable	0·15	4·34	1·56	42·00
5·27	0·66	7·80	4·00	2·54	61·30
4·87	1·13	17·60	3·30	3·20	88·80

It is also apparent that as the chain length of the ester group increases there is a logarithmic increase in surface activity and adsorbability. Comparison of the adsorption data with the results of other workers for the antimicrobial effectiveness of this series of compounds reveals a close parallel in one case. Sabalitschka (1951) tested the germicidal effectiveness of the esters of *p*-hydroxybenzoic acid and the slope of the line log germicidal effect *v.* chain length for his results is 0·33 while that for log A/R *v.* chain length is 0·34. Murrell and Vincent (1950) compared the inhibitory effectiveness of these esters against the growth rate of *A. aerogenes.* The slope of the line log inhibitory effect *v.* chain length was 0·26 which does not show such good agreement with the adsorption data as did Sabalitschka's results. These observations do suggest that for this series of compounds antimicrobial effectiveness is directly related to the degree of adsorption. These findings support the view put forward by Ferguson (1939). He stated that since the molar toxic concentrations in an homologous series change on ascending the series in equal logarithmic steps it can be concluded that they are largely determined by a distribution equilibrium between heterogeneous phases—the external circumambient phase and a biophase. He believed that the equilibrium may be an adsorption equilibrium.

(*b*) *Theoretical Interpretation of Sodium Benzoate Adsorption*

The adsorption data obtained with sodium benzoate at different pH levels indicate that as the acidity increases and the proportion of unionized molecules increases the adsorption of sodium benzoate increases (Table 3).

To determine the proportional contribution to the total amount adsorbed of the unionized and the ionized forms of the molecule, the following formula (suggested by Professor A. E. Alexander) has been used:

$$\text{Amount adsorbed} = (\text{RCOOH})a_1 + (\text{RCOO}')a_2\,, \quad \ldots\ldots(1)$$

where (RCOOH) and (RCOO′) designate the concentrations of the unionized and the ionized molecules respectively, and a_1 and a_2 are the coefficients of adsorption of the unionized and ionized forms respectively.

The calculations have been based on theoretical initial concentrations of 100 mM. The amount adsorbed from the theoretical initial concentrations is plotted against per cent. unionized molecules in Figure 4. The equation of linear regression fitted to these points is:

$$Y = 7{\cdot}43 + 0{\cdot}35x\,. \quad \ldots\ldots\ldots\ldots\ldots\ldots(2)$$

The values of a_1 and a_2 which best fit the experimental points can be found from the regression equation, and, by appropriate substitution in equation (1), $a_1 = 0{\cdot}4243$, $a_2 = 0{\cdot}0743$. The ratio $a_1/a_2 = 5{\cdot}71$.

From these results it can be deduced that under the conditions of these experiments the unionized form of sodium benzoate is 5·7 times as adsorbable as the ionized form.

The data for the adsorption of sodium benzoate at different pH levels support the thesis put forward by Trim and Alexander (1949) that the unionized form of this compound should be more strongly adsorbed than the anion. The fact that the anion is adsorbed to some extent supports the conclusion of Barbour and Vincent (1950) that some activity must reside in the benzoate ion. Unfortunately it is not possible to obtain inhibition data for a particular organism over any but a narrow range of pH and therefore reliable comparisons of inhibition and adsorption data cannot be made. It seems reasonable, however, to conclude that at least part of the explanation of the increased antimicrobial effectiveness of sodium benzoate under acid conditions lies in the greater adsorbability of the unionized form of the compound. The fact that sodium benzoate causes leakage of ultraviolet-absorbing compounds from the cells at low pH levels (see Section IV(*c*)) may still further increase the effectiveness in the high acid range. That this phenomenon is not solely due to increased surface activity is apparent from the fact that the more highly surface-active esters of *p*-hydroxybenzoic acid do not have this effect, even at pH 3·0 in the case of the ethyl ester. However, the absolute amount of sodium benzoate adsorbed was greater than that of any of the esters. The release of ultraviolet-absorbing compounds from the cells may well be the result of a combination of increased cell surface instability at low pH, total amount of compound adsorbed, and surface activity of the compound.

Comparison of the adsorption data for *p*-hydroxybenzoic acid and sodium benzoate (at neutrality) with the inhibitory data recorded by Barbour and Vincent (1950) for these compounds yields some interesting results. The two compounds showed approximately the same adsorbability. The A/R values were 0·102 and 0·074 for *p*-hydroxybenzoic acid and sodium benzoate respectively. The X_{50} values (concentrations giving 50 per cent. inhibition) were quite different. For sodium benzoate the X_{50} value was 7·6 mM and for *p*-hydroxybenzoic acid it was 27·5 mM. Apparently sodium benzoate is much more effective per molecule adsorbed than is the hydroxy compound. Thus adsorbability alone does not guarantee antimicrobial effectiveness.

VI. ACKNOWLEDGMENTS

I am deeply grateful to Professor A. E. Alexander for his helpful advice and to Mr. G. McPherson for his invaluable technical assistance.

VII. REFERENCES

ANTONOFF, G. (1945).—*Arch. Biochem.* **6**: 199.

BARBOUR, R. G. H., and VINCENT, J. M. (1950).—*J. Gen. Microbiol.* **4**: 110.

FERGUSON, J. (1939).—*Proc. Roy. Soc.* B **127**: 387.

MURRELL, W. G., and VINCENT, J. M. (1950).—*J. Soc. Chem. Ind.* **69**: 109.

SABALITSCHKA, T. (1931).—*Pharm. Pr.* **1931**: 173.

TRIM, A. R., and ALEXANDER, E. A. (1949).—*Symp. Soc. Exp. Biol.* **3**: 111.

SOME COMPARATIVE PROPERTIES OF β-GLUCOSIDASES SECRETED BY FUNGI

By M. A. Jermyn*

[*Manuscript received January* 15, 1959]

Summary

Some properties of the β-glucosidases secreted into the medium during shake culture by a number of fungi have been measured. These properties are sufficiently alike to make it probable that the β-glucosidases conform to some common pattern and the biological significance of this observation is discussed.

I. Introduction

A number of Japanese workers have come to the conclusion that glycosidases can be divided into two distinct types, usually designated as *taka* and *emulsin* types from their respective occurrence in "taka-diastase"—a mixture of many enzymes secreted by *Aspergillus oryzae*—and in "emulsin" which is extracted from the seeds of the almond, *Prunus armenica.*

This distinction is clearest when the nature of the competitive inhibitors of various enzymes is considered. Thus (Nisizawa 1951) the β-galactosidases of *P. armenica* and cattle liver are inhibited by aryl glucosides and the gluconate ion, and those of *Zea mays*, *Sesamum indicum* (cereals), *Aspergillus niger*, *Irpex lacteus*, and *Corticium centrifugium* (fungi) are inhibited by aryl galactosides, the galactonate ion, and galactose. Within either group of enzymes, the relative specificity towards a series of substrates does not remain constant from enzyme to enzyme, although the ratio of activity against substrate A to activity against substrate B will be of the same order of magnitude for different members of the group. Very similar conclusions have been reached for β-xylosidase (Morita 1952, 1956) and for β-glucosidase (Ezaki 1940; Horikoshi 1942). *Taka* β-glucosidases are of course inhibited by gluconate, and *emulsin* β-glucosidases by galactonate.

The distinctions set out above do not appear to be absolute and most authors agree in considering the *taka* and *emulsin* types as extremes of a range rather than as clearly defined classes.

Earlier work in these laboratories (Jermyn 1955*b*, 1955*c*) has led to the development of techniques that could be routinely applied to any β-glucosidase to determine its properties. It therefore seemed of interest to check the β-glucosidases of a number of filamentous fungi to see whether they did in fact form a homogeneous group. The fungi were, for the most part, of strains known to be cellulolytic to some degree and were obtained from the U.S. Quartermaster Collection (Reese, Levinson, and Downing 1950).

---

* Division of Protein Chemistry (formerly Biochemistry Unit), C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

The approach used was deliberately made discursive, a few data being obtained for each of a large variety of fungi. This was because no information existed that allowed a small number of exemplars to be chosen for more intensive study. The primary purpose of this work was in fact to discover suitable organisms for such studies.

The inhibition of the action of β-glucosidase of *Stachybotrys atra* (Jermyn 1955*c*) by *ortho*-substitution of aryl glucosides may possibly be a constant feature of β-glucosidases (Veibel and Shih-Lin Yang 1952). The experiment was therefore devised in such a way that the substrates used for checking the relative specificities of the fungal enzymes formed a series of increasing *ortho*-substitution.

II. Enzyme Production

The investigation was simplified by growing the fungi in shake culture at 28°C on a medium that had proved suitable for the extracellular production of β-glucosidases by *S. atra*. Thus a negative result does not mean that a given fungus cannot secrete β-glucosidase under other conditions, nor is the observed order of relative activities of any absolute significance. The medium used is given in Table 1. Its formulation will be discussed in a later paper.

Table 1

MEDIUM USED FOR GROWTH OF FUNGI

Component	Concn. (g/l)	Component	Concn. (g/l)
Soluble starch	10	$CaCl_2$ (anhydrous)	20×10^{-3}
K_2HPO_4 (anhydrous)	6	$ZnSO_4.7H_2O$	2×10^{-3}
NH_4Cl	8	$MnSO_4.7H_2O$	1×10^{-3}
Yeast extract (Difco)	1	Biotin	20×10^{-6}
$MgSO_4.7H_2O$	1		

Fifty ml of this medium in 250-ml Erlenmeyer flasks were inoculated with 1 ml of a spore suspension prepared from a slope culture on malt agar. The flasks were then shaken for 5 days at 28°C at 100 cycles/min, with a 2-in. excursion. The mycelium was filtered off and the culture medium tested for enzymic activity against *p*-nitrophenyl β-glucoside. The results of a survey experiment are shown in Table 2.

To obtain a partially purified concentrate for determining the properties of the enzyme, the filtrate from four flasks was bulked (*c*. 150 ml), cooled, two volumes of cold ethanol were added, and the mixture allowed to stand overnight at 1°C. The precipitate was removed by centrifugation, redissolved in 10 ml of water, and any insoluble matter centrifuged off. This solution was then dialysed in cellulose sausage casing against running tap water for 24 hr in a rotary dialyser. The enzyme in this dialysed concentrate (about 20 ml) was in most cases stable enough to store for a few days at 1°C while it was being used in experiments. In particular, proteinase, as measured by attack on azocasein (Jermyn 1953), was absent in all cases.

TABLE 2

β-GLUCOSIDASE PRODUCTION BY FUNGI GROWN IN SHAKE CULTURE FOR 5 DAYS AT 28°C ON THE STANDARD MEDIUM

Organism	*p*-Nitrophenyl-β-glucosidase Activity of Medium (units/ml)	Organism	*p*-Nitrophenyl-β-glucosidase Activity of Medium (units/ml)
Acremonium sp.	>0·8	*Gelatinosporium* sp.	>0·8
Acrostalagmus cinnabarinus	0·64	*Gliocladium roseum*	0·17
Aspergillus fischeri	>0·8	*Gliomastix convoluta*	0·13
A. nidulans	>0·8	*Helminthosporium* sp.	0·25
A. niger	>0·8	*Hypomyces ipomoeae*	0·52
A. niger mut. *schiemanii*	>0·8	*Memnoniella echinata*	>0·8
A. niveus	>0·8	*Mucor rouxianus*	>0·8
A. ochraceus	>0·8	*Myrothecium roridum*	>0·8
A. oryzae C_1	0·27	*Penicillium citrinum*	>0·8
A. oryzae X_1	0·17	*P. expansum*	>0·8
A. oryzae 292–4847	0·44	*P. frequentans*	>0·8
A. oryzae Cohn-Ehrlich	0·11	*P. funiculosum*	>0·8
A. rugulosus	0·30	*P. griseofulvum*	0·36
A. terreus	>0·8	*P. javanicum*	0·30
A. ustus	>0·8	*P. lilacinum*	>0·8
Botryodiplodia theobromae	0·66	*P. luteum*	>0·8
Brachysporium oosporum	0·17	*P. nigricans*	>0·8
Chaetomium atrobrunneum	>0·8	*P. notatum*	>0·8
C. cochliodes	0·23	*P. piscarium*	>0·8
C. funiculum	0·30	*P. pusillum*	>0·8
C. indicum	0·22	*P. simplicissimum*	>0·8
C. spirale	0·14	*P. soppi*	0·14
C. velutinum	0·01	*Pestalotia palmarum*	>0·8
Cladosporium herbarum	0·37	*Pestalotia virgulata*	>0·8
Curvularia brachyspora	>0·8	*Rhizopus japonicus*	0·42
Curvularia falcata	0·11	*R. oryzae*	>0·8
Curvularia lunata	0·11	*Sordaria humana*	0·21
Curvularia maculans	0·11	*Stachybotrys atra*	0·52
Cylindrocephalum aureum	0·08	*S. atra* var. *brevicaule*	0·57
Fusarium diversisporum	>0·8	*S. atra* var. *lobulata*	0·74
F. moniliforme	>0·8	*S. lobulata*	0·80
F. roseum	>0·8	*Stemphylium consortiale*	>0·8
F. semitectum	>0·8	*Zygodesmus* sp.	0·63
F. solani	>0·8		

Further purification would have involved considerable detailed study of each product and this was not considered justified at the present stage of the work,

TABLE 3

PROPERTIES OF THE β-GLUCOSIDASES FROM A NUMBER OF FUNGI WHICH PRODUCED HIGH ENZYME ACTIVITIES IN THE SURVEY EXPERIMENT (TABLE 2)

Organism	% Activity in Presence of		pH Optimum of *p*-Nitrophenyl-β-glucosidase Action†	Michaelis Constant‡				Relative $V_{\max.}$ (phenyl β-D-glucopyranoside = 1)	
	Gluconate*	Galactonate*		*p*-Nitrophenyl β-D-glucopyranoside	Phenyl β-D-glucopyranoside	*o*-Cresyl β-D-glucopyranoside	2,6-Xylyl β-D-glucopyranoside	*o*-Cresyl β-D-glucopyranoside	2,6-Xylyl β-D-glucopyranoside
Acremonium sp.	0	100	4·8	67	14	§	§	‖	‖
Aspergillus fischeri	9	100	4·7	26	17	19	17	0·11	0·09
A. nidulans	1	98	5·0	8·1	21	58	105	0·07	0·08
A. niger	0	97	4·5	17	180	47	6·2	0·04	0·012
A. niger mut. *schiemanii*	16	100	4·5	42	*c.* 1000	§	§	‖	‖
A. niveus	0	100	5·1	28	*c.* 500	*c.* 1000	§	<1	‖
A. ochraceus	16	100	5·9	13	§	§	§	‖	‖
A. terreus	6	100	4·8	59	250	§	§	‖	‖
A. ustus	15	100	4·8	28	75	§	§	‖	‖
Chaetomium atrobrunneum	2	97	5·3	16	13	§	§	‖	‖
Curvularia brachyspora	1	100	5·1	*c.* 1000	250	300	§	1·10	‖
Fusarium diversisporum	3	98	4·8	33	100	24	§	0·06	‖
F. moniliforme	5	99	4·8	9·2	90	33	100	0·10	0·07
F. roseum	0	96	4·8	19	40	45	§	0·24	‖
F. semitectum	5	99	4·8	5·8	7·7	37	§	0·4	‖
F. solani	6	99	4·8	6·2	90	51	§	0·12	‖

TABLE 3 (*Continued*)

Organism	% Activity in Presence of		pH Optimum of *p*-Nitrophenyl-β-glucosidase Action†	Michaelis Constant‡				Relative $V_{\max.}$ (phenyl β-D-glucopyranoside = 1)	
	Gluconate*	Galactonate*		*p*-Nitrophenyl β-D-glucopyranoside	Phenyl β-D-glucopyranoside	*o*-Cresyl β-D-glucopyranoside	2,6-Xylyl β-D-glucopyranoside	*o*-Cresyl β-D-glucopyranoside	2,6-Xylyl β-D-glucopyranoside
Penicillium citrinum	36	57	4·5	19	180	§	*c.* 500	‖	<0·1
P. expansum	38	60	4·5	12	200	11	§	0·07	‖
P. frequentans	38	76	4·5	12	*c.* 500	§	§	‖	‖
P. funiculosum	22	85	4·5	5·5	60	90	*c.* 250	0·26	0·31
P. griseofulvum	26	91	4·3	17	160	27	*c.* 1000	0·2	<0·6
P. lilacinum	12	52	4·5	14	95	n.d.**	200	n.d.**	<0·4
P. luteum	16	63	4·5	21	100	17	36	0·38	0·08
P. nigricans	49	68	5·1	33	60	65	§	0·17	‖
P. piscarium	29	90	4·5	37	31	65	§	0·14	‖
P. simplicissimum	27	70	4·8	2·6	67	25	§	0·7	‖
Rhizopus oryzae	41	77	4·8	72	95	§	§	‖	‖
Stemphylium consortiale	26	59	5·0	16	43	§	§	‖	‖

* Final concentration of glyconate ion = 10^{-3}M.

† 10^{-3}M substrate in McIlvaine buffers at 28°C.

‡ Determined from Lineweaver-Burk plots of enzyme activity at 28°C and pH 5 in McIlvaine buffer against the substrates indicated. All values to be multiplied by 10^{-5}M to give the Michaelis constant.

§ Hydrolysis of substrate using undiluted enzyme for 3 hr too low to construct satisfactory Lineweaver-Burk plots.

‖ $V_{\max.}$ not determinable (see previous footnote).

** Particular experiment not done.

especially since many of the results of other workers with which those of this study are to be compared have in fact been obtained using culture filtrates directly. Yet it should be emphasized that there is no evidence in any single case that the properties listed are those of solitary β-glucosidases. Were the properties those of only one β-glucosidase, however, consideration of the *S. atra* studies suggest that quite intensive purification would afford little change in the biochemical, as opposed to the physicochemical, properties of the enzyme.

III. METHODS

The methods used in determining aglucone release from *p*-nitrophenyl β-glucoside and from non-chromogenic β-glucosides have already been fully set out (Jermyn 1955*a*, 1955*b*, 1955*c*). The Michaelis constants were determined by constructing Lineweaver-Burk (1934) plots from the data for enzyme activity at a suitable range of substrate concentrations. The relative values of $V_{\text{max.}}$ (reaction velocity at infinite substrate concentration) of β-glucosidase for different substrates were also obtained from the Lineweaver-Burk plots and placed on a common basis by correcting for differing reaction times and enzyme concentrations.

In the experiments where inhibition with D-gluconate or D-galactonate was tested stock solutions of the potassium salts of these ions in the pH 5·0 McIlvaine (citric acid–sodium phosphate) buffer (McIlvaine 1921) used were equilibrated for at least 1 week. Readjustment to pH 5 was usually necessary on the first 2 days for the gluconate solutions. Although the final equilibrium mixture of glyconate ion, free acid, and lactones might not have given the optimal degree of inhibition in either case (Conchie 1954) it was at least stable from experiment to experiment.

The shape of the pH–activity curve for the various enzymes was determined using the *p*-nitrophenyl-β-glucosidase method already described (Jermyn 1955*a*) except that the McIlvaine buffer was varied by steps of 0·3 pH units over the range of values from 3·0 to 8·1.

IV. RESULTS

The results of this investigation are set out in Table 3.

V. DISCUSSION

The way in which β-glucosidases were selected for further study by choosing those showing a high activity against 10^{-3}M *p*-nitrophenyl β-glucoside would undoubtedly lead to a certain bias in favour of enzymes with similar characteristics. Figure 1, which illustrates the hydrolysis of this glucoside by three enzymes with high, medium, and low affinity for the substrate, demonstrates that the enzyme with low affinity, that of *Curvularia brachyspora*, must either have been produced in very high concentrations or have been inherently very active to have shown "high activity" in the survey test. Enzymes with even less favourable characteristics would probably have been passed over, as that of *C. brachyspora* would have been if the concentration of substrate used in the test had been slightly lower. Any final justification of the following conclusions drawn from the inspection of the data in Table 3 would involve individual studies of a large number of fungal β-glucosidases at least as intensive as that already devoted to *S. atra*.

(i) The secretion of β-glucosidase into the medium during shake culture seems to be a general property of filamentous fungi. The species used in this study were a heterogeneous group that happened to be amenable to shake culture under the conditions used and were not selected otherwise than for this characteristic—indeed two of them (*Rhizopus japonicus* and *R. oryzae*) are phycomycetes. Yet in every case β-glucosidase was secreted into the medium, even if not in large amounts.

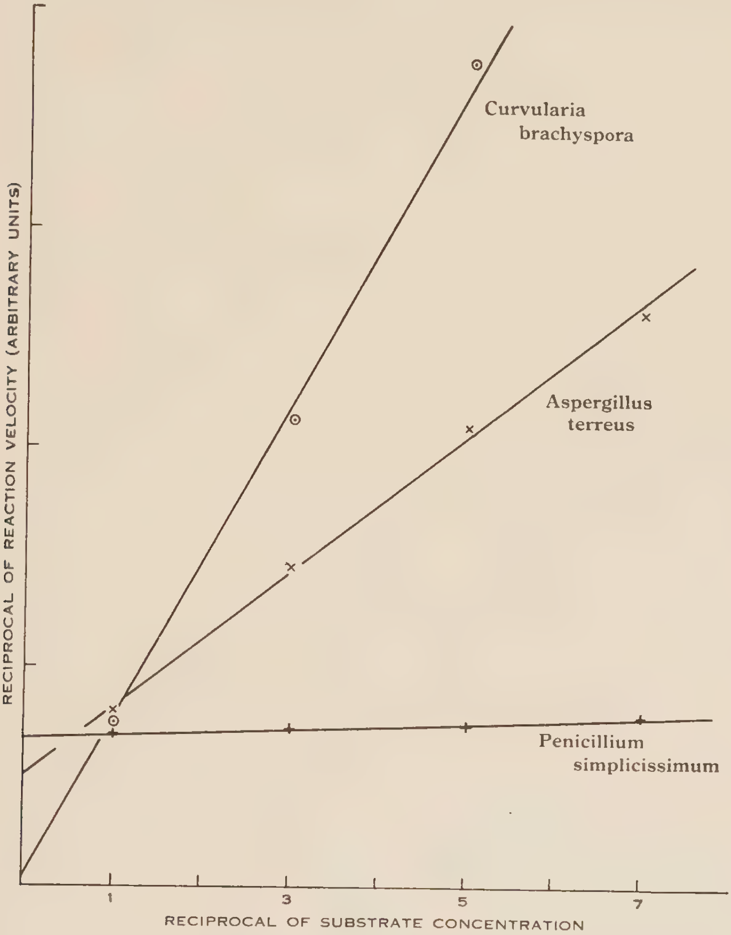


Fig. 1.—Lineweaver-Burk plots for three fungal β-glucosidases acting on *p*-nitrophenyl β-glucoside at 28°C in pH 5·0 McIlvaine buffer. Unit substrate concentration is 10^{-3}M.

The source of the enzyme and the mechanism of its excretion are in all cases obscure. With *S. atra*, aryl β-glucosidase appears to be a normally excreted metabolic product in the absence of the adaptive external production of cellulase and internal production of cellobiase (Thomas 1956). Any understanding of the mechanism of enzyme secretion thus depends in this case on a fundamental understanding of the physiology of the organism. Although it is tempting to believe that the *S. atra* case may be an example of a general phenomenon, a great deal of further work would be needed to establish a hypothesis that hangs on no more than general similarities in enzyme properties, and the escape of cell contents after simple autolysis cannot be excluded.

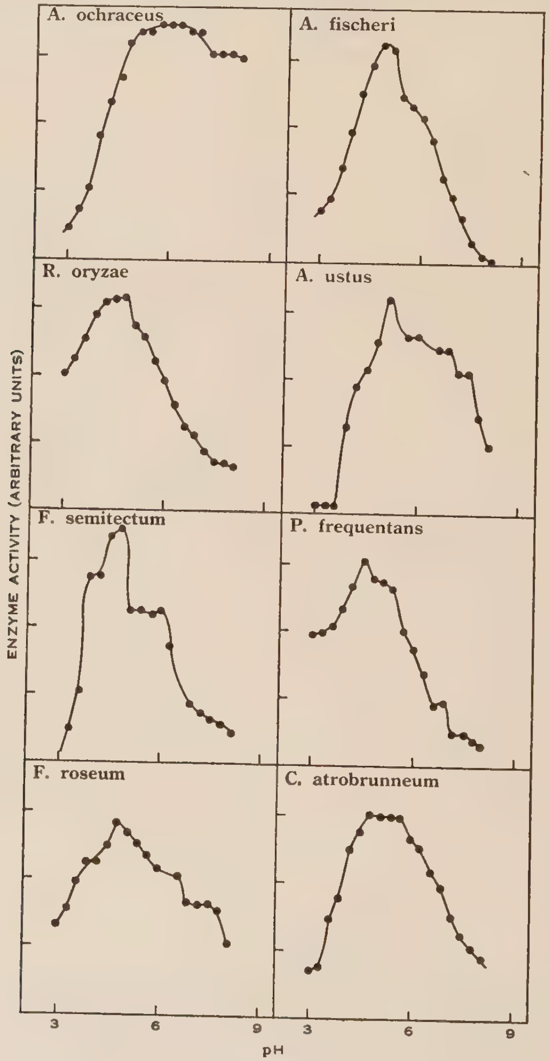


Fig. 2.—Relation between pH and enzymic activity against 10^{-3}M *p*-nitrophenyl β-glucoside for eight fungal β-glucosidases at 28°C in McIlvaine buffers.

(ii) The pH optimum of these fungal β-glucosidases is in the range 4·5–5·0. Where it appears to be higher, e.g. for *Aspergillus ochraceus*, there is a flat-topped pH–activity curve with nearly full enzymatic activity at pH 5·0.

Figure 2 sets out a variety of pH–activity curves to illustrate the similar properties of at least one dominant enzyme in every case; to discuss the variation from the standard form in terms of multiple enzymes or ionic interactions would be unprofitable with such crude preparations.

(iii) All the fungal β-glucosidases examined are strikingly inhibited by gluconate, and are thus *taka* enzymes. However, those of all the Penicillia and two other fungi (*R. oryzae*, *Stemphylium consortiale*) are also inhibited by galactonate, although to a much less degree. This type of inhibition therefore does not provide an absolute differentiation, although the present study does show a characteristic *preferential* inhibition by gluconate that does not seem to intergrade with preferential inhibition by galactonate.

(iv) *Ortho*-substitution in phenyl β-glucoside does not exert any consistent effect on the Michaelis constant which is usually taken to be a measure of the affinity of the enzyme for a substrate. However, it does exert a consistent effect in lowering $V_{\text{max.}}$, which is usually taken as a measure of k_3 in the equation:

$$\text{Enzyme} + \text{substrate} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{enzyme–substrate} \xrightarrow{k_3} \text{enzyme} + \text{products.}$$

In the absence of detailed kinetic studies which will allow the weighing of probabilities, the use of $V_{\text{max.}}$ as a measure of k_3 is no more than an assumption (Ogston 1955), but since this assumption can be used to bring order to the observations on *S. atra* β-glucosidase, it will be tentatively used here.

In the light of a theory of carbohydrase action based largely on the properties of the β-glucosidase of *S. atra* (Jermyn 1957) this lowered value of k_3 must be interpreted as an inhibition of the interaction between bound substrate and bound acceptor. The belief expressed previously (Jermyn 1955*c*) based on the magnitude of Michaelis constants for the *S. atra* β-glucosidase that *ortho*-substitution may interfere with the binding of the substrate by the enzyme appears to be wrongly based on the behaviour of a single enzyme.

(v) There appear to be no correlations between deviations from the various modal properties of the group of β-glucosidases. Variations in such properties as galactonate inhibition, Michaelis constants, pH–activity curves appear to be at random with respect to each other. It seems likely that the fungal β-glucosidases studied have some common pattern with no more than individual variations. The question of whether this is due to a common inherited genetic machinery or is imposed by the nature of the substrate will have to be approached by examining in detail selected and purified representatives of the *taka* β-glucosidases studied here, the *emulsin* β-glucosidases of the higher plants, and of any other groups that may be discovered.

Most of the literature studies on the glycosidases have been carried out at about the level of complexity of the present paper and on preparations of similar "purity"; when even such a classic subject as almond emulsin can be shown to be strikingly inhomogeneous (Baba 1957), none of the inferences which can be drawn by internal or external comparison of existing data can be regarded as more than a guide for further experiment.

VI. ACKNOWLEDGMENTS

I wish to acknowledge the resourcefulness of Mr. A. B. McQuade in inducing very varied organisms to spore profusely on solid medium and grow in shake culture in liquid medium. The tedious routine determinations that defined each value in Table 3 were capably performed by Miss M. Dunne.

VII. REFERENCES

BABA, A. (1957).—*Bull. Agric. Chem. Soc. Japan* **21**: 1166.
CONCHIE, J. (1954).—*Biochem. J.* **58**: 552.
EZAKI, S. (1940).—*J. Biochem., Tokyo* **32**: 91.
HORIKOSHI, K. (1942).—*J. Biochem., Tokyo* **35**: 39.
JERMYN, M. A. (1953).—*Aust. J. Biol. Sci.* **6**: 77.
JERMYN, M. A. (1955*a*).—*Aust. J. Biol. Sci.* **8**: 541.
JERMYN, M. A. (1955*b*).—*Aust. J. Biol. Sci.* **8**: 563.
JERMYN, M. A. (1955*c*).—*Aust. J. Biol. Sci.* **8**: 577.
JERMYN, M. A. (1957).—*Science* **125**: 12.
LINEWEAVER, J., and BURK, D. (1934).—*J. Amer. Chem. Soc.* **56**: 658.
MCILVAINE, T. C. (1921).—*J. Biol. Chem.* **49**: 183.
MORITA, Y. (1952).—*J. Jap. Biochem. Soc.* **24**: 189.
MORITA, Y. (1956).—*J. Biochem., Tokyo* **43**: 7.
NISIZAWA, K. (1951).—*J. Fac. Text. Sericult. Shinshu Univ.* **1**: 213.
OGSTON, A. G. (1955).—*Disc. Faraday Soc.* **20**: 161.
REESE, E. T., LEVINSON, H. S., and DOWNING, M. L. (1950).—*Farlowia* **4**: 45.
THOMAS, R. (1956).—*Aust. J. Biol. Sci.* **9**: 156.
VEIBEL, S., and SHIH-LIN YANG (1952).—*Acta Chem. Scand.* **6**: 1366.

STUDIES ON PLANT PATHOGENIC BACTERIA

I. CULTURAL AND BIOCHEMICAL CHARACTERS

By Rose Mushin,* Jill Naylor,† and N. Lahovary*

[*Manuscript received December* 23, 1958]

Summary

Studies on 119 plant pathogenic bacterial strains belonging to 22 species are described. These include organisms isolated in Australia and type species obtained from abroad.

The collection was maintained by storing the cultures under paraffin oil and in a lyophilized state and both procedures proved to be satisfactory for the survival of most bacteria. Various selective and indicator media were employed and an assessment was made of King's agar and Hartley's glycerol agar for the enhancement of fluorescin and pyocyanin production by *Pseudomonas*. The ability to liquefy a pectin medium was shown to correlate with the ability to decompose plant tissues and both tests were useful for differentiation of *Erwinia* from coliforms and paracolons. Biochemical reactions showed great variation within species and appeared to be of limited value in classification.

I. Introduction

The pathogenicity test is the main criterion for the identification of bacteria suspected of being the aetiological agents of a plant disease. This involves reproduction of lesions following artificial infection of suitable hosts under greenhouse conditions. Occasionally, pathogenicity tests may be performed under controlled laboratory conditions on a part of a plant, such as bean pod, lemon fruit, or on slices of tissue, e.g. potato, carrot. The difficulty of choosing susceptible varieties of the host and suitable conditions indicates the necessity to use additional and practical means of identification of pathogenic bacteria.

This work is concerned with the bacteriological investigation of a number of plant pathogenic species, most of which are endemic in Australia. The study is divided into two parts: in Part I emphasis is placed on methods of maintaining the organisms and on evaluation of cultural and biochemical techniques in classification while Part II is concerned with methods of production of potent antisera and antigenic analysis of various species.

II. Materials and Methods

(*a*) *Cultures*

Type cultures of plant pathogenic bacteria were obtained through the courtesy of overseas workers and local strains were contributed by colleagues or were isolated by the authors of this paper. The suitability of various media for the cultivation

---

*School of Bacteriology, University of Melbourne.

† School of Bacteriology, University of Melbourne; present address: Research Laboratories, Kraft Foods Ltd., Port Melbourne.

(*b*) *Cultural Characters*

(i) *Sheep Blood Agar*.—Most species grew well on this medium but none exhibited haemolytic activity.

(ii) *Deoxycholate Agar*.—This medium allowed good or moderate growth of *Erwinia* within 1–2 days and the intensity of colour of the colonies indicated the active and less-active lactose fermenters. *Agrobacterium* grew well, *Xanthomonas* showed variability and some strains failed to develop, *Pseudomonas* gave good growth except some strains of *Ps. solanacearum* which were inhibited. Gram-positive bacteria are suppressed on this substrate and consequently *Corynebacterium* species failed to grow.

Deoxycholate agar is recommended as a complementary medium for the isolation of most Gram-negative plant pathogenic bacteria from tissues overgrown with contaminants and should be used together with a non-selective medium.

(iii) *Papain Tellurite Agar*.—This medium failed to support the growth of *Corynebacterium* plant pathogens.

(iv) *Fluorescin and Pyocyanin Production*.—Some pseudomonads in our collection lost their ability to produce pigment on brain–heart or nutrient agar medium, also some fresh isolates failed to show colour and therefore various media were tested for the enhancement of fluorescin production.

The following 28 *Pseudomonas* strains, 27 of them plant pathogens, were used in this investigation: *Ps. aeruginosa* (1 strain), *Ps. barkeri* (1), *Ps. cerasi* (2), *Ps. medicaginis* var. *phaseolicola* (5), *Ps. pisi* (2), *Ps. solanacearum* (4), *Ps. syringae* (10), *Ps. viridiflava* (2), and *Ps. fluorescens* (1).

King's agar and Hartley's glycerol agar proved to be the most suitable media for fluorescin production but occasionally pigment was deeper on the first substrate. None of the five *Ps. medicaginis* var. *phaseolicola* strains showed pigment on Hartley's agar but all produced it on Hartley's glycerol agar and on King's agar which contains glycerol. Some strains of *Ps. solanacearum* and one *Ps. viridiflava* did not form pigment in any of the media tested.

The colour of *Ps. aeruginosa* was deeper and a more characteristic blue-green on Hartley's glycerol agar than on King's agar. Eight additional strains of *Ps. aeruginosa* were isolated from the human intestinal tract and again five gave a more characteristic colour on Hartley's glycerol agar.

All pseudomonads were tested for pyocyanin in Hartley's broth with 1 per cent. glycerol and in King's liquid medium by chloroform extraction. An improved technique was the incubation of liquid cultures at 30°C for 48 hr on a shaker. The oxygenated conditions of the medium greatly promoted production of pyocyanin giving a spectacular deep blue alkaline chloroform phase and a brick-red acid phase.

The results of the tests indicated that of the plant pathogenic pseudomonads *Ps. aeruginosa* was the only culture to form pyocyanin.

In conclusion, King's agar proved to be the medium of choice for the enhancement of fluorescin production while Hartley's glycerol agar was slightly less efficient for the demonstration of fluorescin but superior for pyocyanin. These colourless agar media were preferred to the liquid as the opacity of liquid cultures tended to obscure the colour.

(v) *Activity on Pectate Gel Medium.*—Results in Table 2 demonstrate that of the phytopathogens tested all *Erwinia* strains of the soft-rot group liquefied the gel in 2 days; the pH of the medium fell slightly.

Some members of the genus *Xanthomonas*, namely *X. campestris*, *X. carotae*, and *X. vesicatoria*, also decomposed the gel, at times after prolonged incubation, and the pH of the medium did not change. The remaining strains of *Xanthomonas*, *Pseudomonas*, and *Corynebacterium* and also strains of *Bacillus* and *Pseudomonas* of faecal origin did not liquefy the gel and the pH rose slightly.

Table 2

ACTIVITY OF BACTERIA* ON PECTATE GEL AND VEGETABLE SLICES

Species	No. of Strains Tested	Liquefaction of Pectate Gel		Decomposition of Vegetable Slices			
		Degree	Time (days)	Potato		Carrot	
				Degree	Time (days)	Degree	Time (days)
Erwinia aroideae	1	+++	2	+++	2	+++	2
E. atroseptica	2	+++		+++		+++	
E. carotovora	3	+++		+++		+++	
Xanthomonas campestris	3	++,+	3–5	++,+	15–30	++,+	15–30
X. carotae	2	++		++		++	
X. vesicatoria	1	+	20	—		—	
Pseudomonas solanacearum	1	—		+	15	—	
Ps. syringae	1	—		+	30	—	
Aerobacter spp.	2	—		+	15–30	+	20–40
Paracolobactrum spp.	5	—		+	15–30	+	20–40

*The following organisms were inactive: *Agrobacterium tumefaciens* (1 strain), *Corynebacterium flaccumfaciens* (3), *C. michiganense* (3), *Pseudomonas barkeri* (1), *Ps. cerasi* (2), *Ps. medicaginis* var. *phaseolicola* (4), *Ps. pisi* (2), *Ps. solanacearum* (1), *Ps. syringae* (7), *Ps. viridiflava* (1), *Xanthomonas albilineans* (1), *X. incanae* (2), *X. juglandis* (2), *X. phaseoli* (3), *Ps. aeruginosa* (1), *Aerobacter aerogenes* (2), *A. cloacae* (1), *Aerobacter* spp. (13), *Escherichia* spp. (12), *Paracolobactrum* spp. (42), *Ps. aeruginosa*, faecal (2), *Ps. fluorescens* (1), *Bacillus* spp. (2).

Pectate gel was used as a substrate for coliform and paracolon strains. Of these, type cultures of *Aerobacter* (*A. aerogenes* I and II, *A. cloacae*) and our own isolates from human faecal material including 15 *A. aerogenes* I and II strains and 47 paracolons (*Paracolobactrum aerogenoides* I and II, *P. intermedium* I and II, and irregular strains) (Mushin 1949) did not decompose the medium.

Representative strains of the five genera of phytopathogenic bacteria were grown on Burkholder's potato extract medium prior to cultivation on pectate gel. This conditioning did not enhance the pectolytic activity.

TABLE 3

BIOCHEMICAL REACTIONS

A = acid; (g) = gas; V = variable

Species	No. of Strains Tested	Glucose	Lactose	Sucrose	Maltose	Xylose	Salicin	Mannitol	Glycerol	Gelatin	Citrate	Indole	Hydrogen sulphide	Nitrate–nitrite
Agrobacterium tumefaciens	3	A	—	A	A	A	A	V	A	—	V	—	+	—
Corynebacterium fascians	1	A	—	—	A	—	—	—	—	—	—	—	+	—
C. flaccumfaciens	6	V	V	V	V	V	V	V	V	V	—	—	V	—
C. michiganense	3	V		—	—	V	—	—	—	—	—	—	+	—
Erwinia aroideae	2	A	A	A	A	—	A	A	A	—	+	—	+ +	+
E. atroseptica	3	A (g)	A (g)	A (g)	V	A (g)	A (g)	A (g)	V	V	+	—	+ +	+
E. carotovora	3	A (g)	A (g)	A (g)	—	A (g)	A (g)	A (g)	A (g)	V	+	—	+ +	+
Pseudomonas aeruginosa	1	A	—	—	—	A	—	—	A	+	+	—	—	+
Ps. barkeri	1	A	A	—	A	A	—	A	A		+	—	—	—
Ps. cerasi	2	A	A	A	—	A	—	A	A	—	+	—	—	
Ps. medicaginis var. *phaseolicola*	38	A	—	A	—	A		V	A	—	+	—	—	—
Ps. pisi	2	A	—	A	—	A	—	A	A	—	+	—	V	—
Ps. solanacearum	2	A	V	A	V	A	—	V	A	—	+	—	+	V
Ps. syringae	18	A	—	A	—	V	—	A	V	V	+	—	V	—
Ps. viridiflava	2	V	—	—	V	—	—	—		+	+	—	—	
Xanthomonas albilineans	1	A	—	A	A	A	—	A	—	—	+	—	+	—
X. campestris	3	A	A	A	A	A	—	V	V	+	+	—	+ +	—
X. carotae	1	A	A	A	—	A	—	—		—	+	—	+ +	—
X. incanae	1	A	A	—	—	A	—	A	A	+	+	—	+ +	—
X. juglandis	2	A	V	V	A	V	—	—	V	—	+	—	+ +	—
X. phaseoli	4	V	V	V	V	V	—	—	—	V	V	—	+ +	—
X. vesicatoria	2	A	A	A	A	—	—	A	—	V	V	—	+ +	—

With the use of pectate gel a striking separation was obtained for the *Erwinia* soft-rot group from the morphologically, culturally, and biochemically closely related coliform and paracolon strains.

(vi) *Decomposition of Vegetable Slices.*—It was of interest to find out whether the results of pectin liquefaction correlated with the ability to macerate healthy plant tissue. As shown in Table 2, *Erwinia* strains were rapid in tissue decomposition (2 days) while *X. campestris*, *X. carotae*, and one strain of *Ps. solanacearum* and *Ps. syringae* were much slower. All other phytopathogenic strains did not cause any loss of coherence in tissues.

The results of pectate gel liquefaction and vegetable tissue decomposition correlated well in *Erwinia*, while *X. carotae* and *X. campestris* acted more slowly on vegetable slices than on the gel. A few other phytopathogenic strains with feeble activities were irregular in decomposition of gel or digestion of tissues.

Of the 15 *Aerobacter*, 12 *Escherichia*, and 47 *Paracolobactrum* strains tested, two *A. aerogenes* type I and five paracolon strains (four *P. aerogenoides* type I and one type II) softened the tissues, especially potato, after a prolonged incubation of 15–40 days.

Thus the correlation of pectin gel liquefaction and tissue decomposition is more marked for *Erwinia*. A feeble and delayed activity was found with some *Aerobacter* and paracolon strains.

(c) *Biochemical Reactions*

The results of experiments listed in Table 3 allow certain conclusions to be drawn.

Fermentation of carbohydrates was usually delayed and often took place after one or several weeks with the production of acid only, except for *Erwinia* species, most of which evolved acid or acid and gas after 1 or 2 days incubation. Some strains of *Ps. syringae* produced acid after 2 days incubation. *Pseudomonas* and *Xanthomonas* consistently gave negative results in salicin but variable readings were recorded with other carbohydrates for the species tested.

Other biochemical tests in Table 3 showed that all cultures were negative for indole. Hydrogen sulphide production was strongly positive for *Erwinia* and *Xanthomonas*, slight for *Agrobacterium*, and variable for other genera. Growth in citrate medium was consistently positive for *Erwinia* and *Pseudomonas*, negative for *Corynebacterium*, and variable for other genera. Reduction of nitrate to nitrite was positive for *Erwinia*, variable for *Pseudomonas*, and negative for remaining species.

Erwinia strains—soft-rot group—were studied in more detail. As already pointed out, these species were the most active biochemically. In accordance with the classification in "Bergey's Manual" (Breed, Murray, and Smith 1957) a distinction was made between dulcitol-positive strains of *E. carotovora* and dulcitol-negative *E. atroseptica* and *E. aroideae*. The separation of the last two species on gas production proved to be unreliable as some stock strains of *E. atroseptica* were anaerogenic.

Elrod's (1941) differentiation of *Erwinia* on maltose and sorbitol fermentation did not correlate with the breakdown of dulcitol.

A comparison was made between *Erwinia* and the coliform–paracolon group on the basis of "IMViC" reactions (I = indole, M = methyl red, Vi = Voges-Proskauer, C = citrate):

	I	M	Vi	C
E. aroideae	—	—	+	+
E. atroseptica	—	+	—	+
E. carotovora	—	Variable	Variable	+

It can be seen that *Erwinia* species resemble biochemically the organisms of *Aerobacter–Paracolobactrum aerogenoides* and intermediate groups; however, the prompt gel liquefaction and rapid maceration of tissues provided a distinct line of demarcation.

TABLE 4

POD-INOCULATION TESTS ON FRENCH BEANS

Species	No. of Strains Tested	Type of Lesion	Average Size of Lesion (mm)
Corynebacterium flaccumfaciens	6	None	0
Pseudomonas barkeri	1	None	0
Ps. medicaginis var. *phaseolicola*	38	Water-soaked*	3·7
Ps. pisi	2	None	0
Ps. syringae	4	Necrotic	2
Ps. syringae	5	Brown	2·2
Xanthomonas phaseoli	3	Water-soaked*	2
Ps. pyocyanea	1	Brown	2·5
Ps. fluorescens	1	Slight brown	1

*Positive result.

A biochemical variability of strains of the same species was frequently observed. This and the fact that different techniques are frequently used in the bacteriological tests makes it impractical to attempt a comparison of the present results with those listed in Dowson's (1949, 1957), Elliott's (1951), and Bergey's (1957) manuals. With a few exceptions most biochemical tests are of little value in the classification of plant pathogenic bacteria.

(*d*) *Plant Pathogenicity Tests*

These tests are critical in the diagnostic procedure. However, many of our strains were imported from abroad on condition that no pathogenicity experiments would be carried out under greenhouse or field conditions. Accordingly, many tests could not be performed.

The potential bean pathogens in our collection were used for the inoculation of French bean pods. As shown in Table 4 all *Ps. medicaginis* var. *phaseolicola* strains gave characteristic water-soaked "halo blight" lesions after 5–7 days incubation. This test was simple and reliable. *Ps. syringae* produced a different type of lesion and *Ps. aeruginosa* of faecal origin gave lesions similar to those of *Ps. syringae*. Only "halo blight" lesions were considered of diagnostic value.

IV. DISCUSSION

The organisms used in this study were best preserved in a lyophilized state (Gray 1948, 1954) using thick suspensions prepared in 10 per cent. blood in nutrient broth. Storage of cultures under paraffin oil was convenient for routine work. There is variability in the longevity of various strains, for instance *Ps. solanacearum* did not survive one year's storage while Kelman and Jensen (1951) reported viability after 3 years under similar conditions.

Various studies on the demonstration of fluorescin in cultures have been reported and listed by Seleen and Stark (1943). Sullivan (1905) pointed out that the presence of both sulphur and phosphorus was essential for fluorescin formation, and mineral requirements were further investigated by various workers (Burton, Campbell, and Eagles 1948). In this work enriched media with the addition of glycerol were found best.

In decomposition of plant tissues the protopectinases are concerned with the breakdown of cementing material in middle lamellae (Elrod 1942; Sabet and Dowson 1951). The chemical character and macerating ability of pectolytic enzymes secreted by soft-rot bacteria were studied recently (Echandi, van Gundy, and Walker 1957). Some workers did not consider the secretion of pectic enzymes in pure culture to be an indication of bacterial invasiveness (Elrod 1942; Wood 1955). Rudd Jones (1946) found a correlation but pointed out that these two activities are not necessarily the same. The pectin media used by various workers (Elrod 1942; Rudd Jones 1946; Sabet and Dowson 1951; Jacobelli 1953) differed in the origin and type of pectin and in the composition and methods of preparation of media and some critical factor may account for the conflicting results. Tests on synthetic media under controlled conditions would give additional information.

The present results pointed clearly to a correlation between the rapid liquefaction of the pectate gel used and the destruction of plant tissues by *Erwinia* soft-rot bacteria which Dowson (1957) recently classified as *Pectobacterium*. Each of these properties can be used as a demarcation between *Erwinia* and the closely related *Aerobacter*–paracolon group. The rudimentary ability of some coliform and paracolon strains to soften plant tissue due to pectolytic enzymes suggests an interesting line of investigation. More active variants could be selectively isolated to effect a more rapid tissue maceration.

Biochemical variability of various strains of plant pathogenic bacteria showed the limitation of these reactions in the identification of species. Various studies were made on their minimal nutritive requirements (Starr and Mandel 1950) but this did not show a clear distinction between genera and is not practical for routine work.

V. Acknowledgments

The authors wish to thank Professor S. D. Rubbo for helpful criticism in the preparation of this manuscript. They are indebted to Professor Sir Samuel Wadham for assistance in obtaining grants from the Agricultural Research Fund, the A. M. White Trust Fund, and the Henry Gyles Turner Scholarship to support this work.

Some cultures were obtained by the courtesy of the following workers: Dr. W. J. Dowson (Cambridge), Dr. H. Katznelson (Ottawa), Dr. Z. Volcani (Israel), Mr. H. Freeman and Mr. D. E. Harrison (Victoria), the late Mr. R. D. Wilson (New South Wales), Dr. G. C. Wade (Tasmania), and from the Division of Plant Industry, C.S.I.R.O., Canberra. Thanks are expressed to Dr. A. J. Metcalfe, Director-General of Health, for permission to import type cultures.

VI. References

Breed, R. S., Murray, E. G. D., and Smith, N. R., ed. (1957).—"Bergey's Manual of Determinative Bacteriology." 7th Ed. (The Williams and Wilkins Co.: Baltimore.)

Burton, M. O., Campbell, J. J. R., and Eagles, B. A. (1948).—*Canad. J. Res.* C **26**: 15–22.

Dowson, W. J. (1949).—"Manual of Bacterial Plant Diseases." (Adam and Charles Black Ltd.: London.)

Dowson, W. J. (1957).—"Plant Diseases Due to Bacteria." (Cambridge Univ. Press.)

Echandi, E., van Gundy, S. D., and Walker, J. C. (1957).—*Phytopathology* **47**: 549–52.

Elliott, C. (1951).—"Manual of Bacterial Plant Pathogens." (Chronica Botanica Co.: Waltham, Mass.)

Elrod, R. P. (1941).—*Bot. Gaz.* **103**: 266–79.

Elrod, R. P. (1942).—*J. Bact.* **44**: 433–40.

Gray, D. F. (1948).—*Aust. Vet. J.* **24**: 66–9.

Gray, D. F. (1954).—D.V.Sc. Thesis, University of Melbourne.

Jacobelli, G. (1953).—*C.R. Acad. Sci., Paris* **236**: 228–30.

Kelman, A., and Jensen, J. H. (1951).—*Phytopathology* **41**: 185–7.

King, E. O., Ward, M. K., and Raney, D. E. (1954).—*J. Lab. Clin. Med.* **44**: 301–7.

Leifson, E. (1935).—*J. Path. Bact.* **40**: 581–99.

Mackie, T. J., and McCartney, J. E. (1953).—"Handbook of Practical Bacteriology." 9th Ed. (E. & S. Livingstone Ltd.: Edinburgh and London.)

Mushin, R. (1949).—*Aust. J. Exp. Biol. Med. Sci.* **27**: 543–56.

Potter, L. F., and McCoy, E. (1952).—*J. Bact.* **64**: 701–8.

Rudd Jones, D. (1946).—*Nature* **158**: 625.

Sabet, K. A., and Dowson, W. J. (1951).—*Nature* **168**: 605.

Seleen, W. A., and Stark, C. N. (1943).—*J. Bact.* **46**: 491–500.

Starr, M. P., and Mandel, M. (1950).—*J. Bact.* **60**: 669–72.

Sullivan, M. X. (1905).—*J. Med. Res.* (N.S.) **4**: 109–60.

Wilson, G. S., and Miles, A. A. (1946).—"Topley and Wilson's Principles of Bacteriology and Immunity." 3rd Ed. (Edward Arnold & Co.: London.)

Wood, R. K. S. (1955).—Mechanisms of Microbial Pathogenicity. (Sym. Soc. Gen. Microbiol. No. 5.) pp. 263–93. (Cambridge Univ. Press.)

STUDIES ON PLANT PATHOGENIC BACTERIA

II. SEROLOGY

By Rose Mushin,* Jill Naylor,† and N. Lahovary*

[*Manuscript received December* 23, 1958]

Summary

Fifteen species of plant pathogenic bacteria were used for the production of high-titre antisera. The serological relationships were determined for plant pathogenic and other bacteria belonging to the genera *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas* by slide and tube agglutinations and agglutinin-absorption tests. α and β antigens were not important in masking "O" antigens but other factors were found to interfere with some agglutinations.

It was concluded that diagnostic antisera can be useful in the identification of plant pathogenic bacteria, in epidemiological studies, and in screening isolates from plant pathogenicity tests.

I. Introduction

Part I of this series (Mushin, Naylor, and Lahovary 1959) was concerned with the cultural and biochemical characters of plant pathogenic bacteria endemic in Australia and of type cultures obtained from abroad. This paper presents investigations on the antigenic structure of these organisms with the aim of utilizing this character in systematic identification.‡

In some earlier studies of plant pathogenic bacteria often no distinction was made of various antigenic components of the cell. Antisera produced with organisms of poor antigenicity were of low titre, unsuitable for diagnostic work.

The limitation of antigenic analysis as the sole tool of identification is well known in certain genera, and it was also observed that some physiologically unrelated species may share antigenic factors. However, the value of serological characters as a distinguishing feature of plant pathogenic bacteria has been demonstrated (Burkholder and Starr 1948) and these tests have been employed by various workers.

II. Materials and Methods

Agglutination tests mainly with "O" antisera, and with "H" antisera to a much lesser extent, were used in the present investigation. The serological techniques were according to Kauffmann (1954) unless otherwise stated.

---

*School of Bacteriology, University of Melbourne.

† School of Bacteriology, University of Melbourne; present address: Research Laboratories, Kraft Foods Ltd., Port Melbourne.

‡Dr. Mushin would be grateful for cultures of plant pathogenic bacteria isolated in Australia for serological typing.

(*a*) *Preparation of "O" Antisera*

(i) *"O" Antigens for Injections*

To minimize the production of extracellular material, meat infusion or brain-heart (Difco) agar cultures without the addition of carbohydrates were used. Bacteria were harvested as soon as enough growth was available, after 24 hr incubation or longer when dealing with slower-growing organisms.

Adjuvants were introduced in view of the poor antigenicity of some bacteria.

(1) *Steamed* (*St*) *and Roschka's* (*R*) *Antigens.*—Roschka's alcohol–acetone-treated suspensions were standardized to opacity equivalent to 4000×10^6 organisms per ml.

(2) *Roschka's Antigen plus Paraffin Oil plus Tubercle Bacilli Adjuvants* (*R-Par*).—The technique was modified from Freund *et al.* (1948). The suspension was prepared by Roschka's method, the extract being made from 10 ml of saline suspension containing the growth from four petri dishes. Sterile, dried tubercle bacilli (obtained by the courtesy of Dr. A. Pound, Brisbane) were ground to a fine powder using a pestle and mortar. After a few preliminary tests with varying amounts of the reagents the mixtures adopted for further use consisted of 1 ml bacterial suspension, 1 ml "Eucerin" (emulsifying agent), 3 ml paraffin oil, and 2 mg tubercle bacilli. The material was broken up either in a supersonic disintegrator or in an electric homogenizer. The emulsions were stable at room temperature but were warmed prior to injections to reduce viscosity.

(3) *Steamed Antigen plus Sodium Alginate Adjuvant* (*St-Alg*).—Slavin's (1950) method was followed.

(ii) *Course of Injections*

The procedure varied according to the antigen used and the following methods were applied:

(1) *St-Conven.*—Steamed antigen was administered in a conventional manner.

(2) *R-Conven.*—Roschka's antigen was administered in a conventional manner at the earlier stage of this work in six intravenous injections, the first two in 0·2 ml doses and the remainder in 0·5 ml. The first four injections were given within a fortnight and the following doses at weekly intervals. The alternative was a course of six injections of 0·5 ml each at 4–5 days interval.

(3) *St-Shock or R-Shock.*—Steamed or Roschka's antigen was administered by "shock" treatment. In the preliminary experiments the type and dose of antigen and period of injections varied. Finally the procedure was standardized to six intravenous injections of Roschka's antigen within 8 days and bleeding after 3 days. The series of injections at short intervals was referred to as shock treatment.

(4) *R-Par.*—Roschka's antigen with paraffin oil and tubercle bacilli adjuvants, prepared as described above, was used. As a starting point two or three subcutaneous injections of 0·25 ml of this emulsion were administered simultaneously. This was followed by various procedures employed to supply comparative data. In some experiments an additional course of subcutaneous injections with or without the adjuvants was given within 6 weeks or longer. The next step was shock treatment or

the conventional course with Roschka's antigen. Additional details about the techniques used are supplied in Tables 1–3.

(5) *St-Alg*.—Injections of steamed antigen plus sodium alginate adjuvant were administered by Slavin's (1950) technique using the intraperitoneal route. After an interval of about 3 weeks, 0·5 ml Roschka's antigen was given three times in 4-day intervals or by shock treatment.

(*b*) *Preparation of "H" Antisera*

Flagella suspensions were prepared by passaging the bacteria several times through semisolid (0·4 per cent.) agar in Craigie tubes till active motility was induced. Formolized bean-extract broth cultures were injected into rabbits in the conventional manner.

(*c*) *Preparation of α and β Antisera*

Thermolabile surface α and β antigens were prepared with paracolon Fairbrother α strain (Stamp and Stone 1944) and paracolon 30β (Mushin 1949). Formolized nutrient broth cultures were injected into rabbits in the conventional manner.

All sera were preserved with glycerol.

(*d*) *Preparation of Antigens for Tube Agglutination Tests*

(i) *"O" Antigens*.—In preliminary agglutination tests the suitability of steamed, Roschka's, alcoholized, and autoclaved suspensions was compared. The first two suspensions proved to be the most sensitive antigens and for convenience steamed ones were used as a rule in routine work. They were prepared in a concentrated form, formolized, stored, and diluted suitably when required.

Modifications were introduced in the preparation of a few antigens. Some *Erwinia* suspensions were centrifuged lightly to dispose of the deposit showing in control tubes. Trypsin treatment was applied for *Xanthomonas* to digest the mucoid components which were responsible for impaired agglutinability. A trypsin preparation (B.D.H.) in 1 : 1000 final concentration was allowed to act on the antigen for 10 min at 37°C; the mixture was then boiled to inactivate the enzyme.

(ii) *"H", α, and β Antigens*.—These were prepared as previously described.

(*e*) *Agglutination Tests*

Slide agglutination tests were carried out with living suspensions and undiluted "O" antisera as a preliminary and rapid means of demonstrating a qualitative relationship between bacteria. Readings were taken within 1–2 min. α and β tube agglutinations were performed as described by Stamp and Stone (1944) and Mushin (1949). Absorbed antisera were prepared by standard techniques with living suspensions in 1 : 10 dilution and were preserved with "Merthiolate". In dealing with mucoid antigens such as *Xanthomonas*, steaming of the absorbing antigen for 2½ hr was helpful in removing the gummy material which otherwise formed a jelly-like mass with the antiserum.

TABLE 1

METHODS OF PRODUCTION AND TITRES OF C. MICHIGANENSE ANTISERA

The abbreviations used in Tables 1–3 represent the antigen composition and methods used for the course of injections as set out in Sections II(a)(i) and II(a)(ii) respectively

Expt. No.	Course of Injections of Antigens	Titre	Expt. No.	First Course of Injections of Antigens	Time between First and Second Course of Injections (days)	Second Course of Injections of Antigens	Duration of Expt. (weeks)	Titre
1	St-Conven	40	4	St-Alg	17	R-Shock	4	10,240
2	R-Conven	320	5	St-Alg	21	R-Conven	7	640
3	R-Shock	5120 (after 11 days)	6	R-Par*	10	3R-Conven‡	6	640
			7	R-Par	42†	3R-Conven‡	9	2,560
			8	R-Par	10	3R-Conven‡	6	2,560

*Without tubercle bacilli.

†Titre <20.

‡Numbers before the antigen show the number of injections, different to those indicated in the text.

In Tables 4–7 the origin of strains used as antigens are indicated by the following letters: A from Australia, C from Canada, E from England, I from Israel, and U from the United States.

III. Results

(*a*) *Titre of Antisera Produced with Various Techniques*

Attempts were made to produce diagnostic antisera with simple methods but when inadequate titres were obtained more complicated techniques were applied. The merits of various procedures were assessed in relation to a genus or species. Titres of test bleedings were expressed for non-glycerolated sera.

(i) Agrobacterium.—Titres of two antisera prepared with Roschka's antigen from one strain of *A. tumefaciens* administered by the conventional and shock method were 5120.

(ii) Erwinia.—Steamed or Roschka's antigens administered in a conventional manner were used to prepare antisera with two strains of *E. atroseptica*, two strains of *E. carotovora*, and two antisera against one strain of *E. aroideae*. The sera obtained had titres of from 640 to 2560 and both types of antigen were of equal value.

(iii) Pseudomonas.—Antisera were prepared with the following strains: two of *Ps. medicaginis* var. *phaseolicola*, three of *Ps. syringae*, one each of *Ps. barkeri* and *Ps. solanacearum*, and two of *Ps. pisi*. Roschka's antigens were usually more satisfactory than steamed but the highest titre (20,000) was obtained with a steamed suspension of *Ps. medicaginis* var. *phaseolicola*.

(iv) Corynebacterium.—Table 1 shows the experimental data for *C. michiganense*. Roschka's and steamed suspensions applied in a conventional manner gave low-titre sera while shock treatment proved to be more efficient. Good results were obtained with adjuvants but the procedures took a longer time.

Experimental data for *C. flaccumfaciens* antisera are shown in Table 2 and again the superiority of shock treatment with Roschka's antigen was noticed. Earlier experiments with the use of adjuvants followed by shock treatment were more time-consuming. In one exceptional case good-titre serum was obtained with subcutaneous injections.

(v) Xanthomonas.—As shown in Table 3 satisfactory antisera were obtained for *X. carotae* and *X. juglandis* by various techniques. Variable results were recorded with *X. phaseoli*, and *X. campestris* gave only low-titre sera. However, higher titres were shown with Roschka's antigens, especially some old stock suspensions, than with steamed antigens. Working on the assumption that the impaired antigenic sensitivity may be due to the mucoid components of the bacteria, trypsin digestion was applied and a rise in titres was observed. This suggests that the mucoid fraction of the cell may be a critical factor.

(*b*) *α and β Antigens and Antibodies*

α antigens were not demonstrated in cultures of plant pathogenic bacteria and β antibodies were either absent or of a low titre in sera of normal rabbits and therefore of no significance.

TABLE 2

METHODS OF PRODUCTION AND TITRES OF C. FLACCUMFACIENS ANTISERA

Expt. No.	Course of Injections of Antigens	Titre
1	St-Conven	160
2	9R-Shock*	640 (after 17 days)
3	R-Shock	2560 (after 9 days) 640 (after 17 days) 160 (after 24 days)

Expt. No.	First Course of Injections of Antigens	Time between First and Second Course of Injections (weeks)	Second Course of Injections of Antigens	Titre	Time between Second and Third Course of Injections (weeks)	Third Course of Injections of Antigens	Duration of Expt. (weeks)	Titre
4	R-Conven	5	R-Par	160 (after 9 weeks) 20 (after 11 weeks)	11	R-Shock	13	1280
5	St-Conven	1	St-Alg		4	R-Conven	8	1280
6	R-Par	1	2R-Par, R-S/C†	1280 (after 7 weeks) 160 (after 10 weeks)	11	R-Shock	12	5120
7, 8, 9	R-Par	1	R-Par (modified methods)	20–80		R-Shock		5120

*Numbers before antigen show the number of injections, different to those indicated in the text.

†S/C = subcutaneous injection, different to technique indicated in the text.

A few cultures gave weak positive slide agglutinations with β antisera but these were not confirmed in tube tests. Some *X. phaseoli* and *X. flaccumfaciens* cultures agglutinated in β antisera to titres of 256 and 128 respectively, but the reactions were delayed and of granular character and therefore non-specific. β agglutinations of titres up to 40 were recorded in some normal rabbit sera but the level of β antibodies did not increase during immunization.

It will be indicated later that some plant pathogens possessed surface antigens but these were of a different nature to α and β types.

TABLE 3

METHODS OF PRODUCTION AND TITRES OF XANTHOMONAS ANTISERA

Species	Expt. No.	Course of Injections of Antigens	Titre
X. carotae	1	St-Conven	320
	2	R-Conven	1280
X. juglandis	3	R-Shock	2560 (after 11 days)
	4	St-Alg and R-Shock	2560 (after 4 weeks)
X. campestris	6–10	Various (see Tables 1 and 2)	20–160, 1280*
X. phaseoli	11–16	Various (see Tables 1 and 2)	160–640, 2560*

*Trypsin-treated antigens.

(*c*) *Slide Agglutination Tests with "O" Antisera*

In slide agglutination tests of living suspensions with 14 homologous antisera no reaction was observed with several organisms apparently due to the presence of blocking surface antigens. This frequently occurred within the genus *Erwinia* but agglutination was shown when suspensions were boiled to destroy the heat-labile components or by choosing subcultures deficient in surface antigens. Also some *Xanthomonas* and *Ps. syringae* cultures possessed surface mucoid antigens which blocked agglutination with homologous somatic antisera. Distinct agglutinations were obtained with several organisms such as *A. tumefaciens*, *Ps. medicaginis* var. *phaseolicola*, *Ps. syringae*, *Ps. solanacearum* while other bacteria gave non-specific fine granular clumping.

Various species within one genus were found to cross-agglutinate, e.g. strains of *Ps. solanacearum* agglutinated in *Ps. syringae* serum; *Ps. barkeri* and *Ps. viridiflava* in *Ps. solanacearum* serum. Also some bacteria agglutinated in sera prepared with organisms from different genera apparently due to sharing of minor antigens, e.g. between *C. michiganense* and *Ps. pisi*, *X. campestris* and *Ps. syringae*, *X. carotae* and *Ps. syringae*. Frequently reactions were unilateral, not characteristic for species as strain differences were encountered, and of a low enough titre to be regarded as negative in subsequent tube agglutination tests.

Living strains of *A. tumefaciens*, *C. flaccumfaciens*, all *Erwinia*, *X. phaseoli*, *Ps. barkeri*, *Ps. solanacearum*, and *Ps. aeruginosa* did not react in any of the heterologous antisera. However, this may not indicate lack of antigenic relationships because, as previously mentioned, some living organisms, e.g. *Erwinia* were inagglutinable even in their homologous antisera.

It was concluded that results of slide agglutinations with living organisms and "O" antisera can be unreliable and should be confirmed by tube agglutination tests.

TABLE 4

TITRES OF "O" AGGLUTINATIONS OF CORYNEBACTERIUM

Capital letters used in Tables 4–7 are explained in the text (see p. 237)

Antigen		Antiserum from:	
		C. flaccumfaciens U	*C. michiganense* C
C. flaccumfaciens	E	1280	—
	A	1280	—
	U	2560	—
	C	640	—
C. michiganense	A	—	2560
	C	—	5120
	E	—	640
C. fascians		—	—
C. simplex		—	—
Corynebacterium, 7 spp.*		—	—
C. diphtheriae mitis		20	—

*Human and animal pathogens and saprophytes.

(*d*) *Tube Agglutinations of "O" Antigens and "O" Antisera*

The antigenic relationships will be discussed within the appropriate genera and titres will be expressed for glycerolated stock sera. Titres less than 20 were recorded as negative. In all, 50 somatic antisera were produced from 15 bacterial species, namely, *A. tumefaciens* (2 antisera), *C. michiganense* (8), *C. flaccumfaciens* (9), *E. aroideae* (2), *E. atroseptica* (2), *E. carotovora* (1), *Ps. barkeri* (1), *Ps. medicaginis* var. *phaseolicola* (2), *Ps. pisi* (3), *Ps. solanacearum* (1), *Ps. syringae* (3), *X. campestris* (5), *X. carotae* (2), *X. juglandis* (2), *X. phaseoli* (6).

(i) *Serology of* Agrobacterium.—*A. tumefaciens* was represented by three strains, two received from England and one from Canada, and all gave full-titre agglutinations (2560) with two antisera. These strains were antigenically closely related and no cross-agglutinations were recorded with heterologous antisera.

(ii) *Serology of* Corynebacterium.—As shown in Table 4, four strains of *C. flaccumfaciens* and three of *C. michiganense* agglutinated to full or high titre in their homologous antisera and failed to react with heterologous antisera.

No affinity was shown between the above species and *C. fascians*, *C. simplex*, *C. diphtheriae gravis* (2 strains), *C. xerosis*, *C. hoffmanii*, *C. equi*, *C. ovis*, and a *Corynebacterium* sp. from soil. A strain of *C. diphtheriae mitis* gave an insignificant agglutination. Thus the serological distinction of *C. michiganense* and *C. flaccumfaciens* was demonstrated.

Agglutinin-absorption tests were carried out with *C. michiganense* antiserum prepared with a type culture from Canada. Absorption was complete with the homologous and the local strain but the strain from England did not exhaust the serum. Thus, *C. michiganense* strains were found to be serologically closely related in sharing a major group antigen while strains from Australia and Canada possessed an additional factor.

TABLE 5

TITRES OF "O" AGGLUTINATIONS OF ERWINIA

Antigen		Antiserum from:				
		E. aroideae	*E. atroseptica*		*E. carotovora*	
		E	U	A	E	C
E. aroideae	E	1280	160	20	320	160
E. atroseptica	U	20	320	20	160	40
	I	—	—	—	—	—
	A	20	40	640	320	160
E. carotovora	E	20	80	20	640	320
	C	20	80	20	640	640
	I	—	—	—	—	—
Paracolons and coliforms, 28 spp		—	—	—	—	—
Paracolon sp.		10	10	10	40	40
Paracolons and coliforms, 34 spp.						—

(iii) *Serology of* Erwinia (*soft-rot group*).—All strains except two from Israel proved to be interrelated as indicated by agglutination tests with five antisera (Table 5). This group was difficult to examine due to antigenic variation in suspensions prepared from subcultures of the same strain. Also, the readings of the tests were often unsatisfactory because end-point agglutinations were not distinct.

Agglutinin-absorption tests were attempted with a few cultures but this met with little success as controls indicated that absorptions of sera with homologous organisms were not complete. Many variations in time and temperature in the absorption stage were tried and also in the kind and amount of absorbing antigen. Tests were set up with concentrated amounts of the absorbing antigen using steamed suspensions which were prepared from the growth on 64 petri plates. Those sus-

TABLE 6

TITRES OF "O" AGGLUTINATIONS OF PSEUDOMONAS

Numbers in parenthesis refer to number of strains giving a particular reaction

Antigen*	No. of Strains	Antisera from:								
		Ps. medicaginis var. *phaseolicola*		*Ps. syringae*			*Ps. pisi*		*Ps. solanacearum*	*Ps. barkeri*
		A_1	A_2	A_1	E	A_2	C	E	E	E
Ps. medicaginis var. *phaseolicola*	37	80– 160 (6) 320– 640 (11) 1280–10000 (20)	40– 80 (11) 160– 320 (18) 640–2560 (8)	—	—	—	— (22) 20–80 (15)	— (20) 20–80 (16) 640 (1)	— (5)	—
Ps. syringae	17	—	—	— (3) 80 (1) 320–1280 (13)	— (3) 320 (1) 640–2560 (13)	— (3) 320 (1) 640–2560 (13)	—	—	20– 40 (2) — (3)	—
Ps. solanacearum	9	—	—	—	—	—	—	—	40– 80 (2) 160– 320 (3) 640–2560 (4)	—
Ps. pisi	2	160– 320	—	—	—	—	2560	2560	80	—
Ps. barkeri	1	—	—	—	—	—	—	—	—	640

**Ps. cerasi* (2 strains), *Ps. viridiflava* (1), *Ps. aeruginosa* (1), and *Ps. fluorescens* gave negative reactions.

pensions were mixed with 1 ml of serum in four absorption stages of 2 hr each. The difficulties encountered may have been due to the age of the cultures and fresh isolates may give satisfactory results.

A number of paracolons and coliforms tested (Table 5) showed no serological relationship with *Erwinia* except for one paracolon strain. It was noticed that *Erwinia* agglutinated in *X. carotae* serum but not vice-versa, and also a slight reaction was recorded in *Ps. syringae* serum.

(iv) *Serology of* Pseudomonas.—The results of agglutinations of nine *Pseudomonas* species against nine antisera are shown in Table 6. All 37 strains of *Ps. medicaginis*, 13 strains of *Ps. syringae*, one of *Ps. solanacearum*, and one of *Ps. viridiflava* were local isolates while the remainder was imported from abroad.

Ps. medicaginis var. *phaseolicola* (Pmp) and *Ps. pisi*: As some affinity was shown between these two species they will be discussed under one heading. Agglutination tests against two antisera labelled Pmp A_1 and Pmp A_2 with 37 *Ps. medicaginis* strains, including isolates from the same plant, showed variation in titres. *Ps. pisi* strains were identical in reciprocal agglutinin-absorption tests and therefore only one strain and antiserum will be referred to in further examinations. *Ps. pisi* showed reciprocal agglutination with Pmp A_1 but not with Pmp A_2.

In agglutinin-absorption tests it was evident that Pmp A_2 strain had an additional factor, not possessed by Pmp A_1, as there was only partial absorption of Pmp A_2 serum with Pmp A_1 antigen while Pmp A_2 strain completely absorbed Pmp A_1 serum. Tests with the remaining Pmp strains against the absorbed sera showed sharing of a major antigen and presence or lack of minor antigens.

Absorption of Pmp A_1 and Pmp A_2 with *Ps. pisi* did not exhaust these antisera. Absorption of *Ps. pisi* antiserum with Pmp A_1 and Pmp A_2 antigens lowered its titre for *Ps. pisi* although no agglutination was noticed of *Ps. pisi* in Pmp A_2 unabsorbed serum. This will be discussed later.

On the basis of agglutinin-absorption tests the following antigenic patterns can be suggested:

Ps. medicaginis var. *phaseolicola* A_1 strain had factors 1, 3
Ps. medicaginis var. *phaseolicola* A_2 strain had factors 1, 2, 3
Ps. pisi strains had factors 3, 4

Several *Ps. medicaginis* var. *phaseolicola* and both *Ps. pisi* strains cross-agglutinated in *X. carotae* antiserum to a low titre.

Ps. syringae: The majority of strains showed a close serological affinity. *Ps. syringae* A_2 antiserum agglutinated its homologous antigen to a much lower titre than some other strains, thus indicating their antigenic variability. Agglutinin-absorption tests were carried out with three strains of *Ps. syringae*, labelled A_1, E, and A_2, and the corresponding antisera. Strains E and A_2 seemed to be identical and contained an additional factor compared with A_1. Agglutination tests of several *Ps. syringae* strains with the absorbed sera gave similar patterns, thus indicating the presence or absence of minor antigenic factors.

Ps. syringae strains agglutinated in *X. carotae* antiserum; the reactions were unilateral and only a minor antigenic relationship was recorded.

Ps. solanacearum: The nine strains of this species were not homogeneous, as shown in Table 6. *Ps. pisi* and some *Ps. syringae* strains agglutinated in *Ps. solanacearum* antiserum to a low titre.

Other pseudomonads: The remaining *Pseudomonas* strains did not agglutinate in the diagnostic antisera.

Table 7

TITRE OF "O" AGGLUTINATIONS OF XANTHOMONAS

Antigen		Antiserum from:			
		X. campestris E	*X. carotae* E	*X. phaseoli* C_1	*X. juglandis* E
X. campestris	E	40	—	80	—
	E*	640			
	C	40	—	80	—
	I	—			
X. carotae	E	20	640	160	640
X. phaseoli	E	—	—	20	80
	C_1	40	20	640	160
	C_1*			1280	
	C_2	—	—	40	—
	C_3			20	
	U			—	
X. vesicatoria	A	—	—	40	—
X. incanae	A	—	—	20	—
X. juglandis	E	160	320	160	1280
	C	160	160	80	640
X. albilineans	E	40	—	80	—

*Trypsin-treated antigens.

(v) *Serology of* Xanthomonas.—*X. campestris* and *X. phaseoli* gave low-titre sera but antigens exposed to trypsin digestion produced better reactions (Table 7). *X. juglandis* strains were closely related to each other and shared antigens with other members of the genus. *X. carotae* agglutinated to a full titre in *X. juglandis* antiserum, to a significant titre with *X. phaseoli*, slightly with *X. campestris*, and not with antisera from other genera. On the other hand, many organisms reacted to a low titre in *X. carotae* serum.

(e) *Tube Agglutinations of "H" Antigens and "H" Antisera*

Ps. syringae (14 strains) agglutinated to high titres, from 640 to 5120, thus apparently sharing major flagellar antigens. *Ps. medicaginis* var. *phaseolicola* agglutinated to lower titres ranging from 40 to 640 but this may have been due to the difficulty of enhancing motility in some strains.

IV. Discussion

In this investigation several methods were applied to obtain high-titre antisera in rabbits. Antigens in the form of polysaccharide extracts gave good results with *Agrobacterium*, *Erwinia*, and *Pseudomonas*.

Various studies have shown *Corynebacterium* to be poor antigens (Sniezko and Bonde 1943). Woodroffe (1950) used a quick succession of injections with *C. equi* and recorded low-titre sera while Rosenthal and Cox (1953) were more successful with a similar technique. In the present experiments good-titre sera were produced with adjuvants (Freund *et al.* 1948; Slavin 1950). The shock treatment with Roschka's antigen subsequently adopted was equally successful and had the advantage of using a simpler antigen and a shorter time of administration.

The *Xanthomonas* strains are notorious for their mucoid substances whose polysaccharide nature was investigated by various workers (Leach *et al.* 1957). These exudates were found to give non-specific serological reactions and cross-agglutinations (Elrod and Braun 1947*a*, 1947*b*, 1947*c*). In this work the mucoid fractions were removed by trypsin digestion and thus the somatic antigens became more specific.

Slide agglutination tests with living organisms served as a preliminary basis for classification of species within the identified genus. The interpretation of both positive and negative results had to be made with caution and confirmed by tube agglutinations using steamed suspensions to eliminate blocking components.

A. tumefaciens strains showed a close somatic relationship. An extracellular substance in these bacteria was found by Pinkes and Neill (1957) to possess a serological affinity with *Pneumococcus* type 27 and *Leuconostoc mesenteroides* B 512.

The inclusion of plant pathogens in the genus *Corynebacterium* was criticized by Conn and Dimmick (1947) on taxonomic grounds. In the present studies *C. michiganense* and *C. flaccumfaciens* species appeared to be well defined, homogeneous, and not related to other *Corynebacterium* species tested. Similar patterns were described by Rosenthal and Cox (1953, 1954).

The *Erwinia* soft-rot group which comprises strains biochemically similar to coliforms and paracolons showed a marked antigenic variability and cross-agglutinations. This was previously noticed by Elrod (1941). The serological interrelationships of strains confirm Dowson's (1957) suggestion of uniting the three species of the soft-rot group into one. In the present study a minor antigenic relationship was encountered with a paracolon strain while Elrod (1946) recorded an affinity with *Shigella*. With the new knowledge of the antigenic pattern of Gram-negative bacteria it should be possible to bring a serological order into this group on the lines indicated by Kauffmann (1954).

Cross-reactions were recorded in the genus *Pseudomonas* and this was noticed by other workers (Friedman 1953). Of great interest were *Ps. medicaginis* var. *phaseolicola* and *Ps. syringae* strains as most were locally isolated. *Ps. syringae* strains were obtained mostly from beans while one culture with only a slight antigenic affinity to the other strains was recovered from lemon and thus may have displayed a host specificity. *Ps. medicaginis* var. *phaseolicola* strains showed a great heterogenicity and some were related to *Ps. pisi.* Pmp A_2 and *Ps. pisi* did not show relationship in a direct agglutination test but an affinity was demonstrated by agglutinin absorption. It is possible that the distribution of components on the bacterial cell was such that the primary antigen–antibody contact was impaired. Variations in physicochemical behaviour of the organisms may be responsible for the many unilateral reactions encountered in this study.

In the genus *Xanthomonas* the treatment of the polysaccharide envelope of the bacterial cells with trypsin provided specific somatic antigens. This method suggested a promising line of investigation for further studies.

It is concluded that serology cannot replace other bacteriological techniques but it can be very helpful as an additional tool for characterization of species or strains of plant pathogenic bacteria. Serology may be useful in epidemiological studies in tracing the spread of infection and as a rapid means of identification of isolates in plant pathogenicity tests.

V. ACKNOWLEDGMENTS

Thanks to various people for their cooperation with the authors of this work were expressed in Part I of this paper.

VI. REFERENCES

BURKHOLDER, W. H., and STARR, M. P. (1948).—*Phytopathology* **38**: 494–502.

CONN, H. J., and DIMMICK, I. (1947).—*J. Bact.* **54**: 291–303.

DOWSON, W. J. (1957).—"Plant Diseases due to Bacteria." 2nd Ed. (Cambridge Univ. Press.)

ELROD, R. P. (1941).—*Bot. Gaz.* **103**: 266–79.

ELROD, R. P. (1946).—*J. Bact.* **52**: 405–10.

ELROD, R. P., and BRAUN, A. C. (1947*a*).—*J. Bact.* **53**: 509–18.

ELROD, R. P., and BRAUN, A. C. (1947*b*).—*J. Bact.* **53**: 519–24.

ELROD, R. P., and BRAUN, A. C. (1947*c*).—*J. Bact.* **54**: 349–57.

FRIEDMAN, B. A. (1953).—*Phytopathology* **43**: 412–14.

FREUND, J., THOMSON, K. J., HOUGH, H. B., SOMMER, H. E., and PISANI, T. M. (1948).—*J. Immunol.* **60**: 383–98.

KAUFFMANN, F. (1954).—"Enterobacteriaceae." 2nd Ed. (Munksgaard: Copenhagen.)

LEACH, J. G., LILLY, V. G., WILSON, H. A., and PURVIS, M. R. (1957).—*Phytopathology* **47**: 113–20.

MUSHIN, R. (1949).—*J. Hyg.* **47**: 227–35.

MUSHIN, R., NAYLOR, J., and LAHOVARY, N. (1959).—*Aust. J. Biol. Sci.* **12**: 223–32.

PINKES, A. H., and NEILL, J. M. (1957).—*J. Immunol.* **79**: 525–30.

ROSENTHAL, S. A., and COX, C. D. (1953).—*J. Bact.* **65**: 532–7.

ROSENTHAL, S. A., and COX, C. D. (1954).—*Phytopathology* **44**: 603–4.

SLAVIN, D. (1950).—*Nature* **165**: 115–16.

SNIEZKO, S. F., and BONDE, R. (1943).—*Phytopathology* **33**: 1032–44.

STAMP, T. C., and STONE, D. M. (1944).—*J. Hyg.* **43**: 266–72.

WOODROFFE, G. (1950).—*Aust. J. Exp. Biol. Med. Sci.* **28**: 399–407.

THE INFLUENCE OF THE SOIL MOISTURE REGIME ON THE EXPULSION OF THE LARVAL MASS OF THE NEMATODE *ANGUINA AGROSTIS* FROM GALLS

By N. Collis-George* and C. D. Blake†

[*Manuscript received October* 31, 1958]

Summary

The morphology of galls formed on *Agrostis tenuis* Sibth. following invasion of the floret by the nematode *Anguina agrostis* (Steinbuch) Filipjev is considered, and a mechanism proposed for expulsion of the mass of second-stage larvae contained within these galls.

From a consideration of the expulsion of larvae killed by propylene oxide fumigation, of the changes in gall dimensions and cross-sectional outline during expulsion in water, and of a mean temperature coefficient of 1·4 for the swelling process, it is concluded that expulsion is a physical process resulting from the imbibition of water by a matrix in which the larvae are embedded. In this expulsion, movement of individual larvae does not appear to play any part.

A study of galls embedded in artificial soils of known moisture regimes showed that expulsion was controlled by two distinct soil water properties, namely (i) the soil water suction against which the gall imbibes water from the soil, and (ii) the hydraulic conductivity of the soil which permits transmission of water through the soil to the gall. Some evidence is presented to show that osmotic pressure does not influence expulsion and is therefore not comparable with soil water suction.

It is suggested that in the field the environmental conditions for maximal expulsion and movement of larvae occur only during the period when the soil is draining to, and about at, field capacity, and that these same conditions appear to be optimum for the germination of host seedlings and this coincidence is of survival value.

I. Introduction

Bessey (1905), Marcinowski (1909), Leukel (1924), and Goodey (1930, 1933) have studied the biology of the nematode *Anguina agrostis* (Steinbuch) Filipjev, and their conclusions can be summarized as follows:

(i) Second-stage larvae invade *Agrostis tenuis* Sibth. (bent grass) seedlings and are carried upwards as ectoparasites around the growing point by the elongation of the culm. As cells of the primordial floret begin to differentiate, these larvae move from the outside of the culm to the developing tissues of the floret, in which they become embedded and there undergo a rapid maturation.

(ii) This invasion of floral tissue causes a series of histogenic changes, resulting in elongation of the glumes and lemma of the floret, suppression of the lodicules and stamens, and conversion of the ovarial tissues to a dark-coloured, spindle-shaped gall.

---

* School of Agriculture, University of Sydney.

† School of Agriculture, University of Sydney; present address: Biological Branch, New South Wales Department of Agriculture, Sydney.

(iii) Concurrent with these histological and degenerative changes, the nematode larvae mature and the females lay their eggs singly in the surrounding tissues. After a short incubation period the eggs hatch and these larvae are retained within the now matured gall. This falls to the ground as the plant senesces, or if the bent grass seed is harvested the galls are removed admixed with the seed (see Plate 1, Fig. 3).

(iv) The galls are thus the dispersal phase of the disease, and the larvae within provide the inoculum for the next cycle of the disease.

The second-stage larvae emerge from the galls and enter a free-living soil phase during which, if the life cycle is to be continued, the larvae must become associated with a developing seedling host. Field results of Courtney and Howell (1952) show clearly that the larvae cannot exist in the soil for more than one year without access to a host plant which is developing an inflorescence. These authors suggest that the larvae are unable to obtain their nutritive requirements from the soil during this non-parasitic phase and exist at the expense of their body reserves.

II. Experimental Methods and Results

(a) *Morphology of the Gall*

Microtome cross sections were prepared of air-dried mature galls separated from contaminated New Zealand *Agrostis tenuis* seed and were stained with acid haemotoxylin. These sections showed the gall to be composed of a peripheral rind 12–26 μ in thickness, and an extensive cellular medulla containing first- and second-stage larvae tangled in a skein and embedded in a viscous matrix (Plate 1, Figs. 1 and 2). Sections were stained (Hempleman 1940) with dilute toluidine blue (1 part saturated solution of toluidine blue to 80 parts water), methylene blue (1 : 65), and thionin (4 : 20). The staining reactions indicated the matrix to be rich in a mucoprotein. Degradation of the matrix with hydrochloric acid (Rondle and Morgan 1955) yielded glucosamine suggesting the mucoprotein to be probably of animal origin. Female nematodes of the genera *Heterodera*, *Tylenchulus*, and *Rotylenchulus*, for example, lay their eggs in association with a gelatinous secretion, which, on dehydration, affords some protection and organization to the eggs embedded in it. Likewise for *Anguina agrostis* it is suggested that the eggs are laid in floral tissues in association with a gelatinous secretion. When the eggs hatch the matrix persists in the mature gall and the larvae are embedded in it.

(b) *The Mechanism of Larval Expulsion from a Gall*

A number of galls were fumigated (Snyder and Hansen 1947), by placing them together with 0·5 ml propylene oxide in a sealed and moistened 500-ml jar. After 16 hr fumigation at 25°C, each gall was placed in 5 ml distilled water at 25°C, and it was observed that after immersion for 19–23 hr the larvae had been expelled from each gall. Subsequent examination showed that these larvae had been killed by the propylene oxide fumigation.

Bessey (1905) considered expulsion to result from the absorption of moisture by the larvae which then "uncoil and bore their way to freedom". Goodey (1930) observed that the galls of *Anguina tritici* became moist on falling to the ground and suggested that the larvae "break free" of the softened gall presumably by their own activity. But since we have found that larvae of *Anguina agrostis* killed by propylene oxide fumigation are expelled from the gall in the same time as living larvae, presumably these larvae do not take an active part in their expulsion from the gall, and this is probably also true for the larvae of *Anguina tritici* galls.

The maximum lengths and breadths of selected galls were measured with an optical micrometer. Each gall was then immersed in water at 20°C, and the same dimensions were measured over a period of 26 hr. When the length and breadth of each gall were plotted against immersion time the typical swelling pattern shown in Figure 1 was obtained.

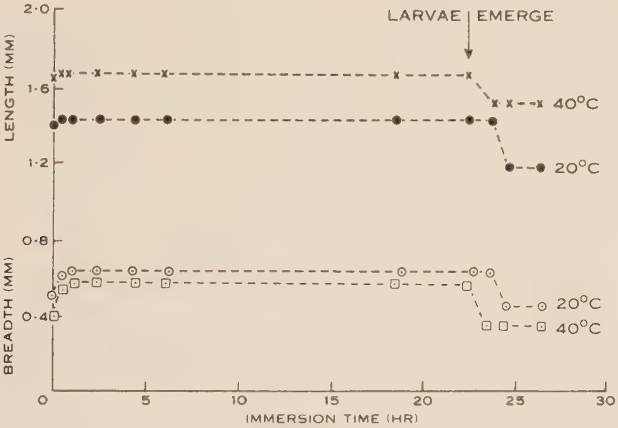


Fig. 1.—Swelling patterns for the length and breadth measurements of two galls during immersion for 26 hr in water at 20 and 40°C.

Within the first 2 hr of immersion the breadth increased on an average by 35 per cent. but the length by only 6 per cent. The larvae were discharged from the end of the gall in a rounded, hyaline mass but occasionally the larvae extrude from the side of the gall. In either case cracking of the gall wall preceded expulsion, which occurred approximately 22 hr after immersion in water at 20°C. However, in a few cases expulsion occurred in as little as 5 hr after immersion, but even in these cases the same swelling pattern was followed. When cross sections of air-dried galls, approximately 20 μ in thickness, were examined, the margins of the sections showed prominent irregular indentations. By comparison with whole galls, these sections became nearly circular within 10 min of immersion in water, as shown in Figure 2.

The rate of increase of breadth at a given temperature compared with the rate of increase at a temperature 10°C lower can be used as a measure of the temperature coefficient, or Q_{10}, of the swelling process of the gall. The mean Q_{10} of gall swelling was determined for batches of galls over the temperature range 18–88°C as $1 \cdot 4 \pm 0 \cdot 5$. A Q_{10} as low as $1 \cdot 4$ suggests that the swelling process as a whole is limited by the rate of a physical reaction (Miller 1938). This evidence, though inconclusive in itself, further supports the hypothesis that expulsion is by a purely physical process.

Since the larvae are embedded in a matrix rich in mucoprotein, a hydrophilic compound, it is suggested that expulsion of larvae results from a hydrostatic pressure developing within the gall consequent on the imbibition of water by the matrix. Once the gall was approximately circular, the limit of elasticity of the rind was reached, and imbibition of further water resulted in the rupture of the rind, and this generally occurred in the terminal region of the gall.

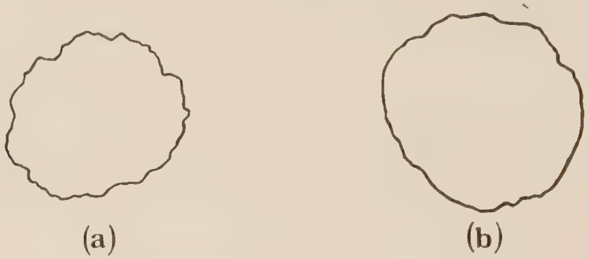


Fig. 2.—Outline of a cross section of a gall 20 μ thick. (*a*) Dry gall; (*b*) outline of the same gall after immersion for 10 min in water at 22°C.

If this hypothesis is correct, it follows that expulsion of larvae is governed by the capacity of the hydrophilic colloid in the matrix to imbibe water from the gall's environment and is independent of active participation of the larvae. Propylene oxide fumigation would not interfere with such an expulsion process, unless it affected the strength of the rind, and this mechanism allows for the expulsion of larvae in distilled water in the absence of a specific chemical stimulus.

(c) *The Influence of Soil Moisture on Expulsion*

(i) *Suction Pressure and Pore Saturation.*—The soil–water relationships of the gall were studied to test the hypothesis of physical expulsion and to evaluate the significance of such a mechanism under field conditions. A range of moisture contents was set up in selected artificial soils by controlling the suction on the water in the porous media, using the tension-plate device shown in Figure 3. Evaporation occurs continuously when using this equipment and thus the horizontal arm controlling suction gradually empties. This arm can be isolated from the sample under suction and refilled from the reservoir and this procedure allows the experiment to run its course with no suction variation, and since the sample of soil is only 1 cm thick the interpretation of the suction value (h cm of water, Fig. 3) is unambiguous.

Onto the sintered-glass disk of 5 μ porosity, 20 ml of "soil" was poured and packed by vibrating the funnel. Fifty galls, selected for size uniformity and absence of ruptures, were inserted into the middle of the soil layer and the soil reconsolidated.

The soil was wetted from below by raising the horizontal arm until a film of water appeared above the soil layer. The arm was then lowered to some height *h* below the sample. This suction was maintained for 72 hr after which galls were separated from the soil by hand, and each examined under the microscope for emergence of larvae.

Distilled water and four artificial soils of sand or alumina were used in these experiments, so that no chemical stimulation of emergence would be expected. The moisture characteristic for drainage (Childs 1940), i.e. the relationship between the moisture content and suction which precisely defines a soil structurally, was determined for each soil using identical consolidation techniques. The moisture characteristics, together with the results of the expulsion experiments, are given in Figures 4(*a*) and 4(*b*). The experiments were carried out in a glass-house where the diurnal temperature varied from 41 to 79°F, over the experimental period of 6 weeks.

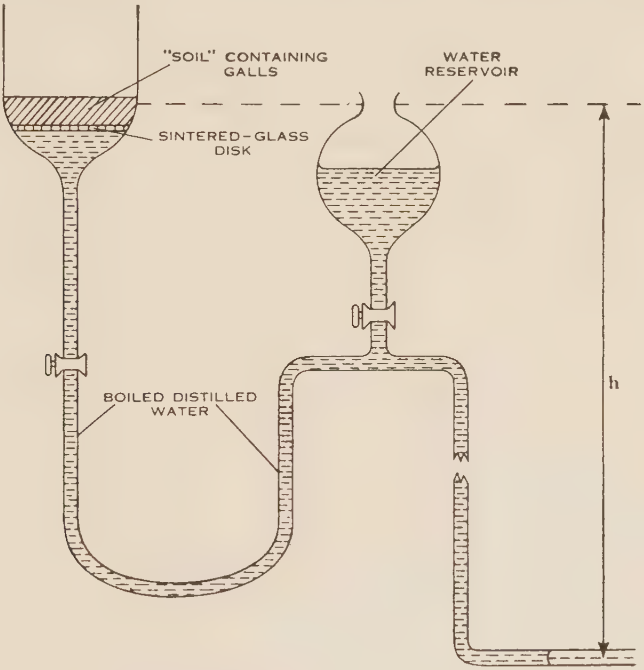


Fig. 3.—Tension-plate equipment for maintaining samples of soil and galls at constant soil water suction; *h*, suction pressure at disk in cm water.

The results of Figure 4 show that the percentage expulsion of larvae at 72 hr invariably decreased as (i) the suction of the soil increased, and (ii) the soil moisture content decreased as evinced by the moisture characteristic. This suggests that there are two distinct phenomena controlling expulsion, and that these are both manifestations of the soil moisture regime. In the 600-grade soil, which remained saturated at all suctions employed in these experiments, less than half of the galls expelled their larvae against suctions greater than 120 cm water, and less than one-tenth against suctions greater than 280 cm of water (Fig. 4). These suctions correspond to work capabilities of $1 \cdot 2 \times 10^5$ and $2 \cdot 8 \times 10^5$ ergs per gram of water absorbed respectively. From Figures 1 and 2, the amount of water which the gall absorbs before the onset of expulsion is found to be 10^{-4} g, and this is equivalent to the extremely small energies of 12 and 28 ergs per gall respectively at the above suctions.

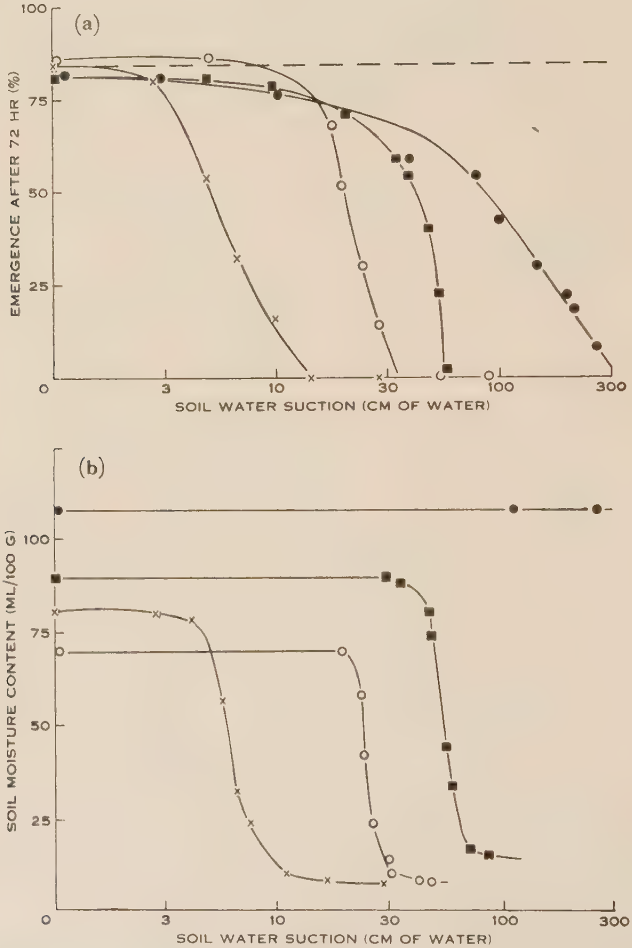


Fig. 4.—(*a*) Effect of soil water suction on emergence of larvae from *A. agrostis* galls. (*b*) Moisture characteristics of soils used in Figure 4(*a*): × 1–2-mm sand (sieved fraction between 1 and 2 mm); ○ 40–50-mesh sand (sieved fraction between 0·4 and 0·3 mm); ■ grade 100 alumina (nominally with lower size of 0·15 mm); ● grade 600 alumina (nominally fraction with lower size of 0·025 mm); ---- osmotic solution (sodium chloride) (in terms of equivalent suction).

Examination of the results for the coarser-grade soils shows a general fall in expulsion with increase in suction as indicated by the suction–energy hypothesis. Simultaneously with a decrease in soil moisture content there is a very marked decline in expulsion. This decline associated with the decreasing moisture content dominates the suction-controlling factor in expulsion and means that the gall is apparently unable to exert the suctions noted above. The interpretation of the decline with decrease in moisture content can be made in terms of the moisture characteristic (Childs and Collis-George 1950*a*). As suction is applied, the largest pores empty first, followed by the smaller pores at higher suctions, until only films and lenses of water remain; at this stage, further appreciable removable of water requires a large increase in suction. Coinciding with this emptying of pores, a marked decrease in the hydraulic conductivity of the system occurs such that at the moisture regime where the majority of the pores are empty, the power of the soil to transmit water becomes extremely small. Thus, in the above experiment, as the pores about a gall empty, the ability of the soil to transmit water to the gall decreases. Furthermore, since the maximum suction that 90 per cent. of galls can exert in the grade 600 soil in 72 hr is less than 280 cm, the suction gradient in the soil moisture about each gall never approaches those established by plant roots in soil moisture where diffusion pressure deficiencies of plant tissues of up to 60 atm, i.e. 6×10^4 cm, have been estimated (Slatyer 1956). In consequence of both a small suction gradient and a low hydraulic conductivity in the vicinity of the surface of the gall, the rate of supply of water to the gall becomes very small. After the gall has absorbed the water from its immediate surroundings, which its energy capabilities allow, it simultaneously establishes a drier soil zone about itself. As a consequence of the resultant low hydraulic conductivity and small suction gradient, this zone virtually prohibits further water moving to the gall so that the gall responds as if situated in a dry environment.

The expulsion of larvae from the gall is controlled ultimately by the moisture content of the soil since suction and hydraulic conductivity are both functions of moisture content. Unfortunately, to define a soil moisture regime by the moisture content alone is not at all specific. To specify completely the experimental conditions at least the moisture characteristic of the soil would be required, since from this relationship the suction is directly derived and, additionally, the variation of hydraulic conductivity with moisture content can be calculated (Childs and Collis-George 1950*b*; Marshall 1958). This argument is supported by the experimental data of Figure 4. For example, if the various soils at 40 cm suction are compared:

(1) In the 600-grade soil expulsion was reduced by 20 per cent. and since all pores are full this reduction can only be attributed to the suction factor.
(2) In the 2–1-mm soil all pores are empty and in this case the hydraulic conductivity factor is dominant.
(3) In the 100-grade soil expulsion was reduced by 50 per cent. and whilst only the larger pores are empty this allows us tentatively to attribute 20 per cent. of the decline to the suction factor and 30 per cent. to the hydraulic conductivity factor.

Two aspects of the experimental procedure need considering for the above conclusions to have any quantitative significance. Firstly, the insertion of a gall into a soil modifies the moisture characteristic in the immediate vicinity of the gall. As a result the moisture characteristic become less "ideal" and approaches that of a heterogeneous medium. Some of the new pores in the vicinity of the gall are larger than those in the pure undisturbed medium, and these exaggerate the effect of pore emptying on the decrease of hydraulic conductivity and cause the expulsion to decrease more rapidly than suggested by the ideal moisture characteristic of Figure 4(*b*). However, this effect is negligible once an appreciable number of pores empty. Secondly, all gall–soil mixtures were in the presence of free water at the beginning of each experiment before the control suction was established. This period was never more than 4 min but to some extent must have enhanced absorption, and hence expulsion, at all suctions. This effect must be small according to the swelling curves of Figure 1. These two imperfections in experimental method were opposite in effect and probably relatively unimportant so that the results shown in Figures 4(*a*) and 4(*b*) are comparable.

(ii) *Osmotic Pressure Effects.*—In the foregoing experiments, soil water suction has been the principal component of total moisture stress, whereas in some soils the osmotic component may dominate. It has been common in soil physics to assume that the effects of osmotic pressure and suction can be regarded as additive, in terms of the total moisture stress. In the wilting of plants for instance, a considerable body of evidence has arisen to indicate that plants permanently wilt and die with total moisture stress in the soil of from 10 to 20 atm, regardless of whether osmotic pressure or suction is the dominant component (Richards and Wadleigh 1952). However, Gingrich and Russell (1957) have shown that, at total moisture stresses less than those of wilting, suction and osmotic pressure are not equivalent in their effect on root growth of oats, and that the growth is greater for a given osmotic stress than for the equivalent suction stress.

Experiments were therefore carried out, using galls of *Anguina agrostis* to determine the relative effects of the osmotic and suction stresses. Samples of 12 representative galls were immersed in sodium chloride solutions, the osmotic pressures of which varied from $0 \cdot 5$ to 10^5 cm (i.e. from 5×10^{-4} to 100 atm). Duplicate experiments showed no difference in the expulsion pattern for any of the osmotic solutions. Galls in these solutions had an average expulsion, after 72 hr, of 82 per cent., whilst the average expulsion in the soil water suction experiments, for suctions approaching zero, was 84 per cent. Expulsion is apparently only sensitive to soil moisture suction and independent of osmotic pressure. It is likely that the gall rind is as freely permeable to sodium chloride as to water and that the calculated osmotic pressure due to concentration is not manifest. It should be noted that although the percentage expulsion remained constant with varying osmotic pressures, the extruded larvae were inactive and showed symptoms of acute plasmolysis in the more concentrated solutions. These experiments provide further evidence that the larvae play no active part in their expulsion from the gall.

III. DISCUSSION

The results indicate that the expulsion of the larval mass from galls of *Anguina agrostis* is dominated by the soil moisture regime of the environment: the wetter the soil, i.e. the lower the soil water suction, the greater the number of galls that eject their larval masses into the soil.

The phenomenon of an organism being controlled by soil water suction and percentage pore saturation as two independent factors has been noted by Parry

(1954) and Wallace (1955). Parry found that lycosid spiders could exert a maximum suction of about 800 cm in a fine-grained porous medium, which had all pores full at this suction, but when placed on coarser media the spiders were progressively less able to extract water as the pores emptied, even though the soil suction was smaller than the limiting value. Wallace, working with the beet eelworm (*Heterodera schachtii*) found that oxygen availability was an indirect factor governing emergence, by its effect on egg hatching, so that maximum emergence did not coincide with saturation. The results of both Wallace and Parry conform to the hypotheses proposed for the expulsion of larvae from galls of *Anguina agrostis*:

(i) That the organism, directly or indirectly, can only overcome a limited soil moisture stress to obtain its moisture requirements.

(ii) That because the organism can only exert a limited suction on its environment, the potential gradient moving water from the medium to the organism can only be small.

Hence if the hydraulic conductivity of the medium is low, due to emptying of the pores, the rate of supply of water to the medium is too small to satisfy its requirements.

Expulsion of larvae at the end of 72 hr has added significance when related to the phenomenon of field capacity, which is generally associated with the moisture regime existing in a soil after 2–3 days drainage following heavy rain or irrigation.

Hence, in the field, moisture regimes would generally have suctions of less than 100 cm only for the 2–3 days succeeding such rainfalls, and the results reported here can be directly applied to the large-scale field emergence of larvae that could occur only during the period of actual rainfall and the subsequent 2–3 days.

Recent work by Collis-George and Sands (1958) on some species of pasture plants suggests that, although germination can occur over a great range of suctions, a more rapid and a higher percentage germination occurs at the lowest suctions, and it is likely that *Agrostis tenuis* would behave similarly. These optimal requirements for seed germination would appear to coincide with the requirements for expulsion of larvae of *Anguina agrostis* and this is considered to be necessary for the continuation of the life cycle of the nematode, since firstly, infection of the bent grass host appears to occur at the seedling stage, and, secondly, the length of the free-living soil phase of the larvae is quite limited.

Wallace (1958*a*, 1958*b*) has demonstrated that, at least for *Ditylenchus dipsaci* and *Heterodera schachtii*, movement of the nematodes through the soil is dependent upon the diameter of the soil pores and upon the arrangement of the water films around the soil particles, and that the interaction of these two determinants reaches its optimum with the onset of drainage, as characterized by the soil–water suction relationships of Figure 4(*b*).

Thus while expulsion of larvae from the gall may be at a maximum in a saturated soil, it is likely, from Wallace's work, that movement of the expelled larvae to a germinating host is favoured in a slightly drier soil and this movement we would expect to take place within the 2–3-day period conventionally associated with field capacity.

Hence the expulsion of larvae from the gall does not require chemical stimulation, but rather the physical activation, represented by the water suction sensitivity of the matrix, appears to be an adaptation comparable in its survival significance to the chemical hatching factor in *Heterodera* spp.

IV. Acknowledgment

Grateful acknowledgment is made to Associate Professor N. H. White, University of Sydney, for introducing one of us (C. D. Blake) to this problem and for supervising the biological investigations carried out.

V. References

Bessey, E. A. (1905).—A nematode disease of grasses. *Science* **21**: 391–2.

Childs, E. C. (1940).—The use of soil moisture characteristics in soil studies. *Soil Sci.* **50**: 239–52.

Childs, E. C., and Collis-George, N. (1950*a*).—The control of soil water. *Advanc. Agron.* **2**: 233–72.

Childs, E. C., and Collis-George, N. (1950*b*).—The permeability of porous materials. *Proc. Roy. Soc.* A **201**: 392–405.

Collis-George, N., and Sands, J. E. (1958).—Germination of pasture species as controlled by soil physical properties. Proc. 3rd Aust. Agrostol. Conf. New England, N.S.W. Vol. 1. pp. 271–9.

Courtney, W. D., and Howell, H. B. (1952).—Investigations on the bent-grass nematode, *Anguina agrostis*. *Plant Dis. Reptr.* **36**: 75–83.

Gingrich, J. R., and Russell, M. B. (1957).—A comparison of effects of soil moisture tension and osmotic stress on root growth. *Soil Sci.* **84**: 185–94.

Goodey, T. (1930).—On *Tylenchus agrostis*. *J. Helminth.* **8**: 197–210.

Goodey, T. (1933).—"Plant Parasitic Nematodes." 1st Ed. pp. 26–30. (Methuen and Co. Ltd.: London.)

Hempleman, L. H. (1940).—Staining reactions of the mucoproteins. *Anat. Rec.* **78**: 197–206.

Leukel, R. W. (1924).—Investigations on the nematode disease of cereals caused by *Tylenchus tritici*. *J. Agric. Res.* **27**: 925–55.

Marcinowski, K. (1909).—Parasitisch und semi-parasitisch an Pflanzen lebende Nematoden. *Arb. biol. Abt.* (*Anst.-Reichsanst.*), *Berl.* **7**: 1.

Marshall, T. J. (1958).—A relation between permeability and size distribution of pores. *J. Soil. Sci.* **9**: 1–8.

Miller, E. C. (1938).—"Plant Physiology." 2nd Ed. (McGraw-Hill Book Co. Inc.: New York.)

Parry, D. A. (1954).—The drinking of soil water by spiders. *J. Exp. Biol.* **31**: 218–27.

Richards, L. A., and Wadleigh, C. H. (1952).—Soil water and plant growth. Ch. 3. In "Soil Physical Conditions and Plant Growth". Agronomy Series 2. (Academic Press Inc.: New York.)

Rondle, C. J. M., and Morgan, W. T. J. (1955).—The determination of glucosamine and galactosamine. *Biochem. J.* **61**: 586–9.

Slatyer, R. O. (1956).—Absorption of water from atmospheres of different humidity and its transport through plants. *Aust. J. Biol. Sci.* **9**: 552–8.

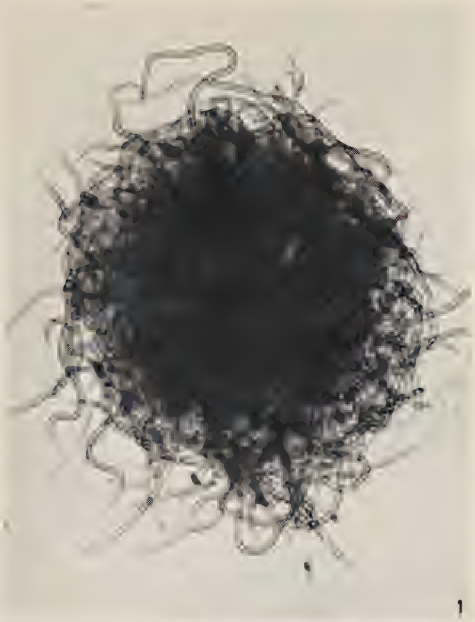
Snyder, H. N., and Hansen, W. C. (1947).—Gaseous sterilization of biological materials for use as culture media. *Phytopathology* **37**: 369–71.

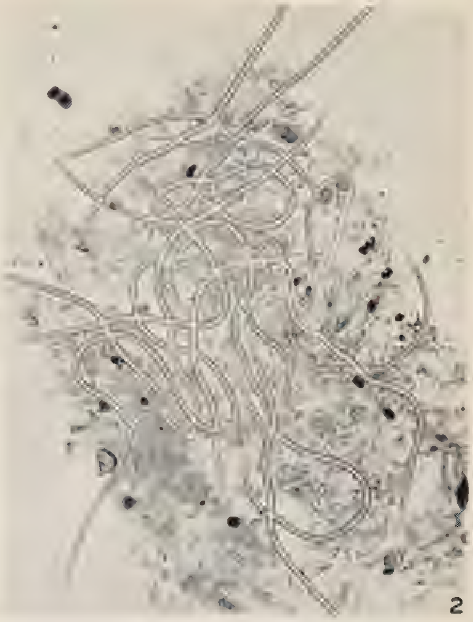
Wallace, H. R. (1955).—Factors influencing the emergence of larvae from cysts of the beet eelworm, *Heterodera schachtii* Schmidt. *J. Helminth.* **29**: 3–16.

Wallace, H. R. (1958*a*).—The influence of pore size and moisture content of the soil on the migration of larvae of the beet eelworm, *Heterodera schachtii* Schmidt. *Ann. Appl. Biol.* **46**: 74–85.

Wallace, H. R. (1958*b*).—A comparative study of the movement in soil of *Heterodera schachtii* Schmidt and *Ditylenchus dipsaci* (Kühn) Filipjev. *Ann. Appl. Biol.* **46**: 86–94.

EFFECT OF SOIL MOISTURE ON NEMATODE EMERGENCE





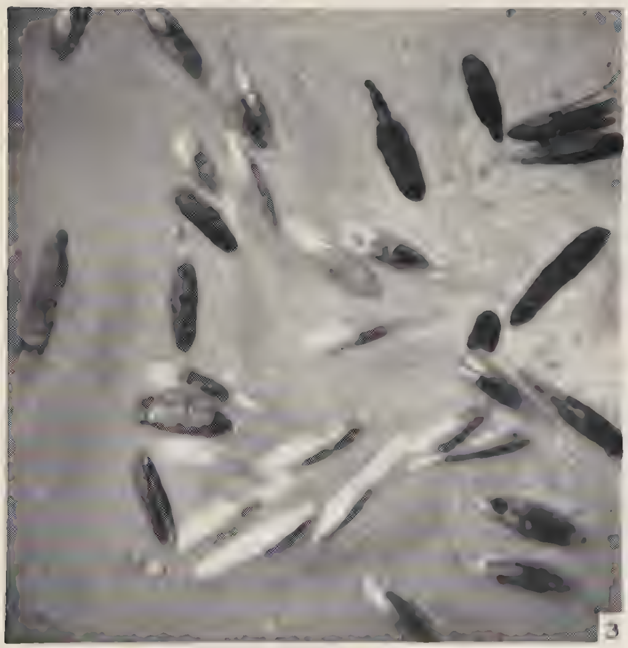


Fig. 1.—Rounded matrix containing first- and second-stage larvae soon after its expulsion from the gall.

Fig. 2.—Larval mass, after expulsion from a gall, showing the viscous material in the matrix.

Fig. 3.—Sample of *Agrostis tenuis* seed, with dark-coloured galls of *Anguina agrostis* admixed.

EXTENSION-GROWTH ACTIVITIES OF SOME *CYCLO*PROPANE DERIVATIVES, A NEW CLASS OF ANTIAUXIN

By N. P. KEFFORD*

[*Manuscript received April* 14, 1959]

Summary

2,3-Dihydrobenzofur-2,3-yleneacetic acid, 1,2-dihydro-1,2-naphthyleneacetic acid, and *cis*- and *trans*-2,3-dihydrothionaphthen-2,3-yleneacetic acid have been shown to competitively inhibit auxin-induced growth of *Avena* coleoptile sections, and thus are antiauxins. *trans*-2-Phenyl*cyclo*propanecarboxylic acid, 2,3-dihydrothionaphthen-2,3-yleneacetic acid dioxide, and 1,2-3,4-tetrahydronaphth-1,2-3,4-di(yleneacetic acid) were inactive towards coleoptile growth.

The structural difference between these antiauxins and related auxins is discussed with reference to structural requirements for antiauxin activity.

I. INTRODUCTION

The molecules of many substances possessing auxin activity contain the following structural features: (1) a ring system, (2) a double bond in a ring, (3) a side chain on one of the doubly bound carbon atoms, (4) an unsubstituted carbon atom *ortho* to the side chain, (5) a carboxyl group (or a structure equivalent to a carboxyl group) on this side chain, (6) a particular space relationship between the ring and the carboxyl group (Thimann 1951).

McRae and Bonner (1953) have shown that molecules lacking in one of the features (4), (5), or (6) above, but with all other features intact, will competitively inhibit auxin-induced extension growth and thus qualify for definition as antiauxins (Tukey *et al.* 1954). In this study, molecules in which the double bond requirement (2) is replaced by a *cyclo*propane ring are investigated for growth activity in the *Avena* section extension test. Other molecules, in which a double bond in the side chain of an auxin has been replaced by a *cyclo*propane ring, have also been investigated. The *cyclo*propane ring is a strained structure and, in this situation, is probably the closest structural approach to a double bond that can be made. Most of the *cyclo*propane derivatives used here have been synthesized by the addition of ethyl diazoacetate to carbon–carbon double bonds of heterocyclic systems (Badger *et al.* 1958; Badger, Rodda, and Sasse 1958); unfortunately no addition product is formed with indole and thus the *cyclo*propane analogue of 3-indolylacetic acid (IAA) could not be tested.

II. MATERIALS

Samples of the following compounds have been supplied by Professor G. M. Badger, University of Adelaide:

(I*a*) *cis*-2-phenyl*cyclo*propanecarboxylic acid, m.p. 105°C (lit., m.p. 105°C).
(I*b*) *trans*-2-phenyl*cyclo*propanecarboxylic acid, m.p. 88–90°C (lit., m.p. 91°C).

---

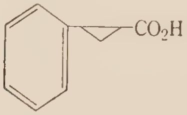
* Division of Plant Industry, C.S.I.R.O., Canberra.

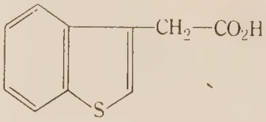
(II) 2,3-dihydrobenzofur-2,3-yleneacetic acid, m.p. 182°C (lit., m.p. 181°C).

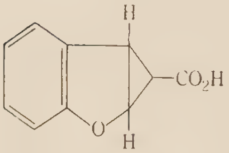
(III) 1,2-dihydro-1,2-naphthyleneacetic acid, m.p. 164°C (lit., m.p. 165–166°C).

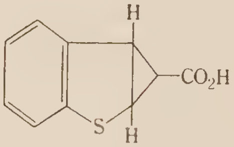
(IV) 1,2-3,4-tetrahydronaphth-1,2-3,4-di(yleneacetic acid), m.p. 282°C (lit., m.p. 286°C).

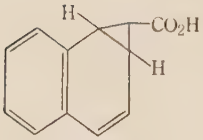
(V) 3-thionaphthenylacetic acid, m.p. 108–110°C (lit., m.p. 109°C).

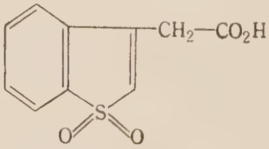

cis (Ia) ; trans (Ib)

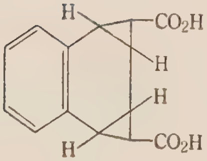

(V)

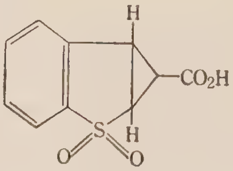

(II)


cis (VIa) ; trans (VIb)


(III)


(VII)


(IV)


(VIII)

(VI*a*) *cis*-2,3-dihydrothionaphthen-2,3-yleneacetic acid, m.p. 182°C (lit., m.p. 182°C).

(VI*b*) *trans*-2,3-dihydrothionaphthen-2,3-yleneacetic acid, m.p. 145–148°C (lit., m.p. 148°C).

(VII) 3-thionaphthenylacetic acid dioxide, m.p. 265°C (lit., m.p. 267°C).

(VIII) 2,3-dihydrothionaphthen-2,3-yleneacetic acid dioxide, m.p. 241°C (lit., m.p. 241°C).

All samples were tested for homogeneity by paper chromatography in butanol ammonium carbonate, using methyl red as indicator. Only *trans*-2-phenyl*cyclo*propanecarboxylic acid was slightly contaminated. The geometrical configurations of the two 2,3-dihydrothionaphthen-2,3-yleneacetic acids have not been unequivocally proven. But Badger, Rodda, and Sasse (1958) present evidence for the acid, m.p. 148°C, being the *trans*-acid. In other cases the isomer present could not be specified, but all samples contained only one isomer.

III. Methods

Series of concentrations of the above substances were tested alone and in mixtures with a series of concentrations of IAA in the *Avena* coleoptile section extension test. The method of McRae and Bonner (1953) was followed except in the following details: in preliminary tests, the growth of 3-mm coleoptile sections over 20 hr was measured, and in tests for use in kinetic studies, 6-mm sections were grown for 10 hr, during which period growth was proportional to time. The methods for the treatment of results have also been fully described by McRae and Bonner (1953).

Table 1

AVENA COLEOPTILE SECTION EXTENSION IN SOLUTIONS OF THE TEST COMPOUNDS LISTED

Increments in section length are expressed as a percentage of the increment obtained in buffer plus sucrose alone. The latter increment was approximately 10 per cent. of the original length

Compound under Test (formula number)	Concentration (M):								
	3×10^{-7}	6×10^{-7}	10^{-6}	3×10^{-6}	5×10^{-6}	10^{-5}	3×10^{-5}	5×10^{-5}	10^{-4}
I*a*			100	140		220	320		290
I*b*			100	100		90	110		140
II	90		100	60		110	80		60
III	100		100	90		40	80		70
IV						100			100
V	110	130	160	230		430			
VI*a*			110			102			60
VI*b*	90		100	120		70	90		70
VII					90	110		85	100
VIII	80		120	120		120	120		120

IV. Results

In Tables 1 and 2 are shown the results of preliminary tests to establish the general nature of the growth activity of the substances.

trans-2-Phenyl*cyclo*propanecarboxylic acid (I*b*), 2,3-dihydrothionaphthen-2,3-yleneacetic acid dioxide (VIII), and 1,2-3,4-tetrahydronaphth-1,2-3,4-di(yleneacetic acid) (IV) were found not to influence coleoptile section extension or the extra extension induced by added IAA. 3-Thionaphthenylacetic acid dioxide (VII) at 10^{-4}M slightly inhibited IAA-induced growth and it has been shown also to inhibit seed germination (Schlesinger and Mowry 1951). *cis*-2-Phenyl*cyclo*propanecarboxylic acid (I*a*) and 3-thionaphthenylacetic acid (V) promoted coleoptile section growth.

thus confirming previous claims (Crook, Davies, and Smith 1937; Veldstra and van de Westeringh 1951), based on other test tissues, that these substances were auxins.

2,3-Dihydrobenzofur-2,3-yleneacetic acid (II), 1,2-dihydro-1,2-naphthyleneacetic acid (III), and *cis*- and *trans*-2,3-dihydrothionaphthen-2,3-yleneacetic acid (VI*a* and VI*b*) were inactive alone (Table 1) and inhibited IAA-induced growth (Table 2). The four substances were then tested with IAA under the conditions required for the application of the kinetic treatment of McRae and Bonner (1953) to the growth results. As is shown in Figure 1, the criteria for competitive inhibition of IAA-induced growth were met for each substance. These substances can thus be called antiauxins.

Table 2

AVENA COLEOPTILE SECTION EXTENSION IN SOLUTIONS OF MIXTURES OF THE TEST COMPOUNDS LISTED AND IAA

Increments in section length are expressed as a percentage of the increment obtained in buffer plus sucrose and IAA of the appropriate concentration. In an optimal concentration of IAA the increment was approximately 800 per cent. of the increment in the absence of IAA

Compound under Test (10^{-4}M)	IAA Concentration (M):							
	5×10^{-8}	10^{-7}	2×10^{-7}	3×10^{-7}	5×10^{-7}	8×10^{-7}	10^{-6}	5×10^{-6}
I*b*	120		105		105			90
IV		95	100	100	105		95	90
VII		90	75		70	85	85	95
VIII	100			85	110			105

V. Discussion

It has been shown that replacement of a double bond, adjacent to the side chain, in the auxins 1-naphthylacetic acid, 3-thionaphthenylacetic acid, and 3-benzfuranylacetic acid by a *cyclo*propane ring produces antiauxins (III, VI*a*, VI*b*, II). Of the two *cyclo*propane derivatives (IV, VIII) that did not exhibit antiauxin activity, one was not an analogue of an auxin (VII) and the growth activity of the other parent compound, 1,4-naphthyldiacetic acid, has not been investigated. If the replacement of the double bond by a *cyclo*propane ring had been the only structural change that had occurred, then another instance of the creation of antiauxins by the elimination of one structural feature essential for auxin activity could be added to those discussed by McRae and Bonner (1953). But in the *cyclo*propane derivatives, the carboxyl group was attached to a fused ring carbon atom rather than a side chain carbon atom and the *ortho* hydrogen atoms were not equivalent to those in the parent auxin molecules.

McRae and Bonner (1953) found that weak auxins could competitively inhibit IAA-induced growth, but there is no evidence for the substances II, III, VI*a*, or VI*b* being auxins (Table 1).

Some auxins such as *cis*-cinnamic acid have a double bond in the side chain. The replacement of this double bond by a *cyclo*propane ring produces another auxin, *cis*-2-phenyl*cyclo*propanecarboxylic acid, as has been demonstrated here for *Avena* sections and by Veldstra and van de Westeringh (1951) for split pea epicotyls.

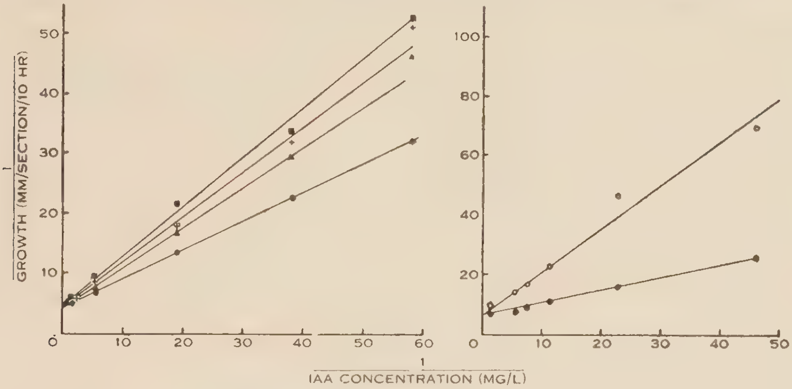


Fig. 1.—Inhibition of 3-indolylacetic acid (IAA)-induced *Avena* section growth by some *cyclo*propane derivatives. Data plotted according to Lineweaver-Burk treatment as a test for competitive inhibition, which requires intersection of the lines at the vertical axis. The greater the slope of the lines the greater has been the inhibition of IAA-induced growth. The treatments were:

- ● IAA alone.
- ▲ IAA plus 2,3-dihydrobenzofur-2,3-yleneacetic acid (II), 5×10^{-5}M.
- + IAA plus *trans*-2,3-dihydrothionaphthen-2,3-yleneacetic acid (VI*b*), 5×10^{-5}M.
- ■ IAA plus 1,2-dihydro-1,2-naphthyleneacetic acid (III), 2×10^{-5}M.
- ○ IAA plus *cis*-2,3-dihydrothionaphthen-2,3-yleneacetic acid (VI*a*), 2×10^{-4}M.

This result might suggest that, in a side chain, a double bond and a *cyclo*propane ring are equivalent. This is not supported by the finding that replacement of the double bond in the antiauxin *trans*-cinnamic acid by a *cyclo*propane ring produces the growth-inactive *trans*-2-phenyl*cyclo*propanecarboxylic acid.

VI. Acknowledgments

Thanks are due to Professor G. M. Badger, who, with his colleagues, synthesized the compounds. I am particularly grateful to Dr. Jenneth M. Sasse for checking the purity of the samples.

VII. References

Badger, G. M., Christie, B. J., Rodda, H. J., and Pryke, J. M. (1958).—Polynuclear heterocyclic systems. XIII. The reaction of ethyl diazoacetate with naphthalene and its heterocyclic analogues. *J. Chem. Soc.* **1958**: 1179–84.

Badger, G. M., Rodda, H. J., and Sasse, J. M. (1958).—The reaction of ethyl diazoacetate with thionaphthen. *J. Chem. Soc.* **1958**: 4777–9.

CROOK, E. M., DAVIES, W., and SMITH, N. E. (1937).—Synthetic plant growth hormones. *Nature* **139**: 154–5.

MCRAE, D. H., and BONNER, J. (1953).—Chemical structure and antiauxin activity. *Physiol. Plant.* **6**: 485–510.

SCHLESINGER, A. H., and MOWRY, D. T. (1951).—Benzothiophenes and their 1-dioxides. *J. Amer. Chem. Soc.* **73**: 2614–16.

THIMANN, K. V. (1951).—The synthetic auxins: relation between structure and activity. In "Plant Growth Substances." (Ed. F. Skoog.) (Univ. of Wisconsin Press.)

TUKEY, H. B., WENT, F. W., MUIR, R. M., and VAN OVERBEEK, J. (1954).—Nomenclature of chemical plant regulators. *Plant Physiol.* **29**: 307–8.

VELDSTRA, H., and VAN DE WESTERINGH, C. (1951).—Plant growth regulators XX. Structure-activity (5). 2-Phenylcyclopropane-1-carboxylic acids. *Rec. Trav. Chim. Pays-Bas* **70**: 1127–35.

STUDIES ON "HAEMIXODOVIN", THE PIGMENT IN THE EGGS OF THE CATTLE TICK *BOOPHILUS MICROPLUS* (ACARINA: IXODIDAE)

By K. C. Bremner*

[*Manuscript received February* 24, 1959]

Summary

The pigment present in the eggs of the cattle tick *Boophilus microplus* is shown to be a chromoprotein made up of a water-insoluble globulin conjugated with the prosthetic group protohaem. The name "haemixodovin" is proposed for the pigment, and its spectral absorption characteristics have been described.

The physiological significance of this haemoprotein in the eggs is discussed, and evidence is presented which indicates that, whereas the protein moiety of the molecule is utilized as a food reserve by the developing larva, the haem grouping is probably a functionless inclusion derived directly from the bovine haemoglobin of the parent tick's diet.

I. Introduction

The first report on the nature of the pigment responsible for the brown colour of tick eggs was made by Wigglesworth (1942), who noted the presence of alkaline haematin in the eggs of *Ornithodorus moubata* (Murray) and *Ixodes ricinus* (L.). More recently, Riek (1959), in the course of studies on toxins present in the eggs of the cattle tick *Boophilus microplus* (Canestrini), observed that a pigmented protein component was present in aqueous and saline extracts of the eggs. Riek figures electrophoresis patterns of the egg extracts and refers to the pigment as fraction F_6.

Since Wigglesworth's (1942) studies gave no indication that the egg pigments he studied were protein-bound, the following investigations were made in an attempt to define the chemical nature of the pigment in the eggs of *B. microplus*, and to assess its metabolic fate.

II. Experimental

(*a*) *Solubility Characteristics of the Egg Pigment*

Aqueous extracts of eggs of *B. microplus*, which had been laid no longer than a week, were prepared by grinding eggs and distilled water in a mortar in the proportion of 1 g eggs to 2 ml water. The extract was centrifuged at 1200 *g* for 30 min, and 8 ml of the supernatant was subjected to zone electrophoresis in a starch-supporting medium as described by Kunkel and Slater (1952). Electrophoresis was carried out in an 0·05M phosphate buffer of pH 11·0, and a voltage of 10 V/cm was applied for 17 hr. The brown pigmented section was then cut from the starch block and eluted with phosphate buffer.

---

*Division of Animal Health and Production, C.S.I.R.O., Veterinary Parasitology Laboratory, Yeerongpilly, Qld.

This solution was then diluted to 200 ml with distilled water, and glacial acetic acid was added drop by drop with stirring until a dense brown precipitate settled out. The pH of the clear supernatant was then 5·1, and this was presumed to approximate the isoelectric point of the pigment. After allowing the mixture to stand for 30 min, most of the supernatant was decanted, and the remaining suspension centrifuged at 1200 *g* for 15 min. The supernatant was removed and the precipitate was shaken with 10 ml of distilled water and again centrifuged, but no pigment was found to have dissolved. After two washings with 10 ml of distilled water, the precipitate was shaken with 10 ml of 0·2 per cent. sodium chloride solution, and complete solution of the pigment was obtained. Presumably sufficient inorganic ions to carry this protein into solution are present in the eggs when they are extracted with distilled water. The fact that dialysis of aqueous egg extracts against distilled water results in precipitation of the pigment supports this view.

TABLE 1

ABSORPTION SPECTRA OF CRYSTALLINE PROTOPORPHYRIN ESTER AND PORPHYRIN ESTER PREPARED FROM B. MICROPLUS EGG PIGMENT

Positions of maxima (mμ) and ratios of intensities (in parenthesis) of the visual absorption bands of samples of porphyrin in dioxane, the intensity of band IV being taken as 1·0

Pigment	Band IV	Band III	Band II	Band I
B. microplus porphyrin methyl ester	504 (1·0)	538 (0·80)	576 (0·465)	631 (0·359)
Crystalline protoporphyrin methyl ester	504 (1·0)	539 (0·784)	576 (0·441)	631 (0·374)
Protoporphyrin methyl ester*	503 (1·0)	537 (0·790)	575 (0·463)	630 (0·381)

*Stern and Wenderlein (1934).

Tests on the pigment in solution in 0·2 per cent. sodium chloride showed that it remained in solution after the addition of two volumes of 0·4N ammonia solution, but was precipitated by the addition of an equal volume of ethanol and by half saturation with ammonium sulphate. The pigment was coagulated by heating the solution to 100°C. From this evidence it was concluded that the protein component of the pigment was of the globulin type (Mitchell 1946).

(*b*) *Spectrophotometry of the Egg Pigment*

Since Wigglesworth (1942) has reported the presence of alkaline haematin in the eggs of the ticks *O. moubata* and *I. ricinus*, and in view of the marked red-brown pigmentation of clarified extracts of *B. microplus* eggs, it seemed likely that the prosthetic group of the egg pigment was of the haematin type. Accordingly, *Boophilus* eggs were extracted with ethyl acetate–acetic acid (3 : 1) and the extract was examined for haem and free porphyrins (cf. Dresel and Falk 1956). No free porphyrins were present, but spectroscopic examination indicated the presence of haem. A portion of the extracted haem was taken to dryness and converted to pyridine haemochromogen; readings of the absorption spectrum in a Beckman spectrophotometer (model DU) showed maxima at 557 and 525 mμ, identical in position to those of a haemochromogen made from recrystallized protohaem under the same conditions. Thus there is little doubt that the egg pigment prosthetic group is protohaem.

To confirm this identification, a further portion of the extracted haem was converted to porphyrin as follows: The dried haem was dissolved in glacial acetic acid, and iron removed by the ferrous acetate–acetic acid method of Warburg and Negelein (1932). The porphyrin so formed was transferred to ether (which had been washed with ferrous sulphate and water to remove peroxides), washed with water, extracted into 4N hydrochloric acid, and returned to fresh ether. It was then dried and esterified by standing in methanol-5 per cent. sulphuric acid at 1°C for 20 hr. The porphyrin ester was then transferred to chloroform, washed with water and then with 2N ammonium hydroxide to remove any unesterified porphyrin, and finally washed with water.

The porphyrin ester was then taken to dryness and purified by chromatography in benzene on a column of Al_2O_3 (grade V) (Nicholas 1951). Benzene eluted most of the ester from the column and this was taken to dryness and dissolved in purified dioxane (Eigenberger 1931). The absorption spectrum of this solution was read on the Beckman spectrophotometer, and compared with the spectrum of a sample of known crystalline protoporphyrin methyl ester read under the same conditions. The correspondence of these two absorption spectra, shown in Table 1, confirms that the prosthetic group of the egg pigment was protohaem.

Studies on the spectrophotometry of the intact egg pigment were carried out on purified preparations obtained by eluting the pigmented band from the filter-paper strips with M/15 phosphate buffer, pH 7·8, after concentrated egg extracts had been subjected to electrophoresis on filter paper for 3 hr in the same buffer with an applied voltage of 10 V/cm (Kunkel and Tiselius 1951). Absorption curves of this preparation (Fig. 1) showed a maximum at 617 mμ, while less well-defined maxima occurred at about 530 and 500 mμ. Intense absorption was displayed with a maximum at 405 mμ in the region of the Soret maximum of pigments containing an intact porphyrin ring (Lemberg and Legge 1949). A further band of intense absorption was manifest in the ultraviolet with a maximum at 277 mμ.

Reduction of the pigment with sodium dithionite resulted in the bands at 617, 530, and 500 mμ being replaced by distinct absorption bands with maxima at 562 and 534 mμ, while the Soret maximum was displaced to about 420 mμ (Fig. 1). Accurate readings were not obtained at wavelengths below 400 mμ, because of interference by the reducing agent. Addition of a few drops of 2N sodium hydroxide to the reduced solution resulted in the immediate formation of a haemochrome with absorption maxima at 557 and 525 mμ (Fig. 2). Passage of carbon monoxide through a solution of the pigment previously reduced by dithionite resulted in the formation of a carboxy compound whose absorption curve (Fig. 2) is very similar to that of carboxyhaemoglobin, but with absorption maxima at 567 and 538 mμ.

Boophilus eggs suspended in distilled water were placed in a spectrophotometer cuvette of 1 mm path length and the absorption spectrum of this preparation over the wavelength range 450–650 mμ was found to be identical with that of the electrophoretically purified pigment extract at pH 7·8. Thus there is little doubt that the pigment does exist as a haemoprotein compound in the intact egg.

Finally, absorption curves were obtained of solutions of the pigment in phosphate and borate buffers covering the pH range 5·5–11·0, and it was found that the absorption spectrum remained unaltered over the pH range examined. This marked stability of the compound despite such changes in pH is very similar to that reported by Fairley (1941) for methaemalbumin.

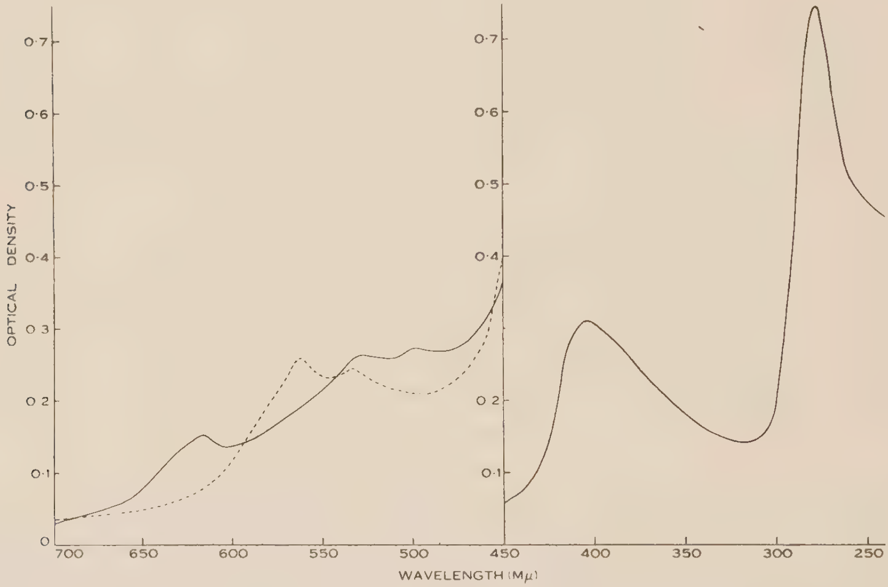


Fig. 1.—Absorption curves of *B. microplus* haemoprotein in phosphate buffer, pH 7·8, before (————) and after (– – – –) addition of sodium dithionite. Readings of absorption at wavelengths below 450 mμ were made on a 1 in 8 dilution of the pigment extract.

(*c*) *The Fate of Ingested Host Iron*

In view of the occurrence of an iron-containing protein in the eggs of *B. microplus*, and since the diet of the female tick includes large amounts of iron in the form of bovine haemoglobin, it was considered of interest to trace the fate of ingested host iron in female ticks. Sections of ovipositing ticks were cut and histochemical tests for the presence of free iron (Pearse 1953) were applied, but none could be demonstrated. Therefore, iron estimations on tick eggs and on engorged female ticks were made by the method of King (1948).

Forty fully engorged female ticks were collected from the same bovine host and were washed three times by shaking in distilled water. The iron content of 20 of these ticks was estimated immediately, and found to be 2·54 mg Fe. The remaining 20 ticks were maintained in an incubator at 30°C and 90 per cent. relative humidity until oviposition was completed 16 days later. The iron content of the parent ticks was then found to be 2·40 mg Fe, while that of the 2·29 g of pooled eggs laid was 0·13 mg Fe. Thus, the total iron content of the eggs plus the parent ticks was 2·53 mg Fe, and 5·1 per cent. of the iron ingested by the adult female ticks had been transmitted to the eggs. Presumably the majority of this transmitted iron is incorporated in the haem of the egg pigment.

The site of the iron remaining in the tick after oviposition was then investigated. Two groups of 10 fully engorged ticks were collected as before, and both groups were allowed to complete their egg laying. The eggs were discarded, and the iron content of one group of ticks was estimated to be 1·24 mg Fe. Each of the remaining 10 ticks was then injected subcutaneously with iron-free *n*-hexanol to expand the shrivelled cuticle and to harden the internal organs, thus allowing ease of dissection. The gut of each tick was then dissected out and the total iron content of the pooled guts was found to be 1·23 mg Fe.

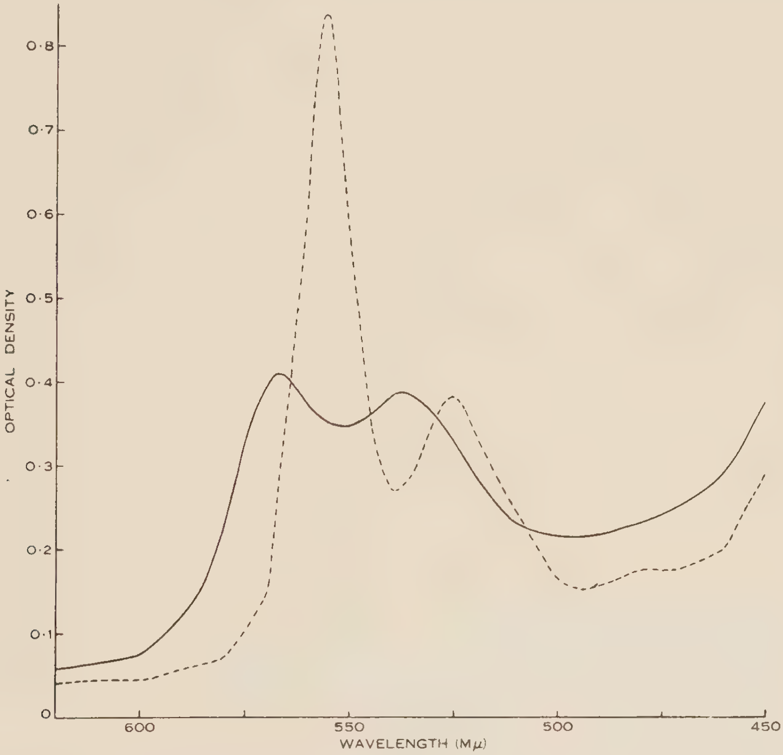


Fig. 2.—Absorption curves of *B. microplus* egg pigment haemochrome (– – – –) and the carboxy compound (————).

Thus, it seems that the ingested iron which is not transmitted to the eggs is merely retained in the lumen of the gut, or in the gut-wall cells. This is in accord with the findings of Wigglesworth (1942) that in *I. ricinus* the contents of the gut and diverticula consist of a core of unaltered blood with a covering of granular black haematin, and the cells of the gut wall contain numerous granules of haematin.

(d) *Ultimate Fate of the Egg Pigment*

The high concentration of pigmented protein in the eggs of *Boophilus* suggests that the pigment may be a major food source for the developing larval tick. Microscopic examination of a newly hatched larva revealed that its sacculated gut was filled with reddish brown fluid. This fluid is undoubtedly entrapped yolk, around which the embryo has grown, and which has the function of sustaining the larva until it attaches to a host. As incubated larvae aged, increasing amounts of a black granular material were deposited around the walls of the larval gut. Extraction of incubated unfed larvae 4–5 weeks old with 0·9 per cent. sodium chloride solution yielded a pigmented extract which has absorption characteristics identical with that of solutions of the haemoprotein extracted from eggs. After extraction of the larval residues (two to three times) until no further pigment could be extracted by the salt solution, subsequent extraction with 0·05N sodium hydroxide solution readily dissolved the black granular material present in the larval residues, yielding a reddish purple supernatant. Passage of this alkaline extract through cellulose powder resulted in adsorption of a pink compound, and a greenish brown compound passed through unadsorbed. This brown solution displayed an absorption spectrum similar to that of alkaline haematin (King and Delory 1945), and readily formed a pyridine haemochrome with absorption maxima at 557 and 525 mμ after addition of sodium dithionite and pyridine. This suggests that the granular deposit in the larval gut contains a haematin-like substance.

The changes with time in the concentration of haemoprotein and haematin in eggs and larvae were followed in eggs laid by engorged ticks collected on the same day from an infested steer. These adults were incubated at 29°C and 90 per cent. relative humidity, and 1 week after the commencement of oviposition the pooled eggs were weighed into 1-g samples. One sample was extracted by grinding with 10 ml 0·9 per cent. sodium chloride solution and the resulting suspension was centrifuged at 9000 *g* for 10 min. The supernatant was removed and the residue stirred with a further 10 ml of salt solution and again centrifuged. This procedure was repeated with 5 ml of salt solution, and the supernatant after centrifugation then appeared colourless. The volume of the pooled supernatants was made up to 30 ml with salt solution, and this saline extract contained all the haemoprotein originally present in the 1-g sample of eggs.

The deposit remaining in the centrifuge tube was then extracted similarly with 0·05N sodium hydroxide solution. These supernatants were also pooled and made up to 30 ml, and the extract was filtered three times through Whatman No. 42 filter papers to remove by adsorption any of the unidentified pink compound present (it was not found in egg extracts, but appeared later in extracts of larvae). This alkaline extract contained all unbound haematin present in the original sample. An index of the amount of pigment present in each extract was obtained by reading the optical density at 530 mμ, a wavelength at which both *Boophilus* egg pigment and haematin absorb light strongly.

The remaining samples of eggs were returned to the incubator, and approximately on each succeeding week one sample was removed and extracted with saline and alkaline solutions as before. The optical density of each extract was determined at 530 mμ.

Nineteen days after the commencement of oviposition larvae commenced to emerge from the eggs, so that extracts prepared 1 and 2 weeks after commencement of oviposition were of eggs, the third extract was of mixed eggs and larvae, and subsequent extracts were of larvae. Larvae began to die during the ninth week and no further extracts were prepared after this time. The amount of haemoprotein extracted from samples decreased rapidly during the first 4 weeks (Fig. 3), and was accompanied by a steady increase in the amount of haematin recovered. These changes occurred during the period of development of the larvae within the eggs, and during hatching. From the fourth to the seventh week, when the larvae were relatively dormant, there was only a slight fall in the haemoprotein concentration of larval extracts, and a similarly slight increase in the concentration of haematin. The first larval deaths were observed on the 58th day of the experiment. The optical density at 530 mμ of the saline extract of larvae on the following day was 0·240, compared with 0·330 10 days earlier. This decrease in haemoprotein was accompanied by a very slight decrease in the corresponding haematin concentrations.

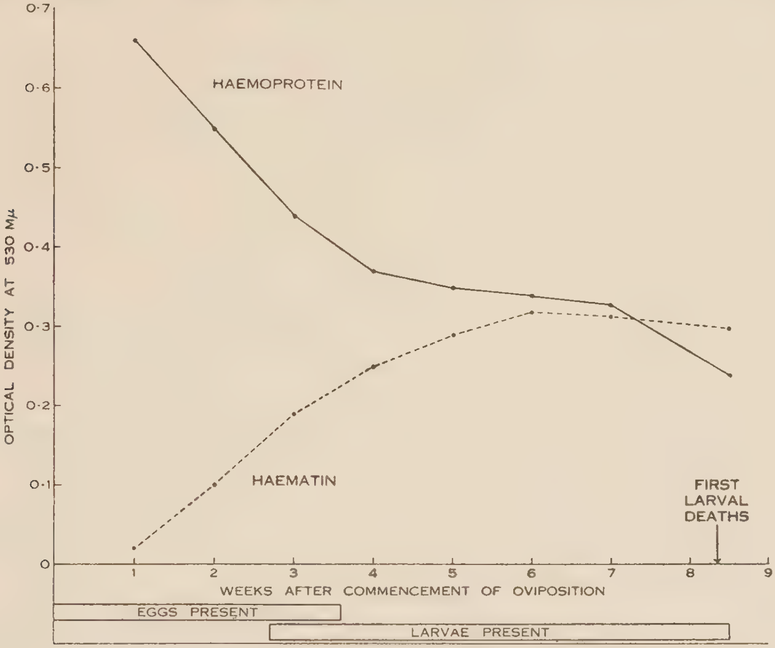


Fig. 3.—Age of sample plotted against the optical density of haemoprotein and haematin solutions extracted from standard samples of *B. microplus* eggs and larvae.

During the incubation of unfed larvae, deposits of ivory-coloured urinary excreta, predominantly guanine (Enigk and Grittner 1952), collected on the walls of the sample tubes. No pigment could be extracted from this solid excreta by sodium hydroxide solution.

When larvae 4–5 weeks old were mounted in 0·05N sodium hydroxide solution and examined microscopically, a reddish purple colour similar to that of the alkaline extracts was seen to develop in the gut caeca. This indicates that the unidentified pink compound and haematin present in alkaline extracts are in fact extracted from the gut contents or the gut wall. The association of the pink pigment with haematin suggests that it may be a breakdown product of haematin. Alkaline extracts of aged larvae prepared as before were passed through a chromatographic column of cellulose powder (length 4 cm, dia. 2 cm) in order to isolate this pigment.

Elution of the adsorbed pink material by 0·05N sodium hydroxide solution yielded a bright pink solution with absorption maxima at 541, 359, and 270 mμ. On addition of sodium dithionite the solution slowly decolorized, and no haemochrome was formed following the addition of pyridine to the reduced solution. During titration of a sample with 0·1N hydrochloric acid, the pink colour changed to violet below pH 7·2, the solution then exhibiting absorption maxima at 552 and 380 mμ, with a plateau in the curve between 340 and 360 mμ. This colour change was reversible.

No colour could be extracted from acid, neutral, or alkaline solutions of the pigment by ether, chloroform, or carbon tetrachloride, either before or after standing in boiling water for 1 hr. No colour change occurred in these solutions during heating. Neither acid nor alkaline solutions fluoresced in ultraviolet light.

On allowing an acid solution of the pigment to stand overnight, an amorphous blue-violet solid precipitated. Evaporation to dryness of an acid solution over a steam-bath also yielded an amorphous blue-violet solid. This was washed several times with distilled water, the washings were discarded, and the deposit was again dried. This solid proved insoluble in acetone, ether, chloroform, carbon tetrachloride, dioxane, methyl cyanide, and methanol, but readily dissolved in 0·05N sodium hydroxide solution to give a pink solution displaying the characteristics of the original eluate.

As the pigment was suspected of being a bile pigment, Van den Bergh's test and Gmelin's test (Lemberg and Legge 1949) were applied to neutral solutions. Both were negative. Similarly, an attempt to obtain a colour reaction by condensation with *p*-dimethylaminobenzaldehyde (Prunty 1945) yielded only a fine black precipitate.

III. Discussion

These findings suggest that the pigment responsible for the characteristic colour of the eggs of *B. microplus* is a conjugated protein with a water-insoluble globulin as its protein moiety, and protohaem as its pigmented prosthetic group. The rather flat appearance of the absorption curve of the pigment suggests that the iron atom in the complex is in the ferric state. A general property of ferriprotoporphyrin complexes is their less-marked absorption peaks compared with the corresponding ferroprotoporphyrin compounds (Lemberg and Legge 1949). The distinct peak in the red region of the spectrum also supports this assertion. The name "haemixodovin" is proposed for this pigment in order to indicate both the source of the protein and the nature of its prosthetic group. It is quite probable that similar haemoproteins will be found in the eggs of other species of ixodid ticks.

It seems almost certain that the haem of haemixodovin is derived from the bovine haemoglobin ingested by the parent female tick. As no free iron could be demonstrated in any tick tissue it would seem that bovine haematin is not broken down in the gut of the tick to provide free iron for incorporation into the haemixodovin of eggs. Rather, it is probable that haem is split free from the ingested bovine haemoglobin and some proportion of it absorbed as such from the gut into the haemolymph, and transferred in combination with a protein (possibly as haemixodovin) to the ovaries where it is incorporated into the yolk of developing eggs. The results of iron analyses suggest that approximately 5 per cent. of the ingested bovine haem could be transferred into eggs as haemixodovin. Wigglesworth (1942) has drawn attention to the presence of a pigment in the haemolymph of the engorged sheep tick *I. ricinus* which exhibits absorption bands similar to those of ferrihaemalbumin, and also to those of *B. microplus* haemixodovin. Later work by Lees and Beament (1948) with *O. moubata* has shown that large haemoprotein molecules can readily pass from the haemolymph through the shell layer of ovarian eggs into the yolk.

It has not been possible to assign any function to the intact pigment. As the iron atom is in the ferric state it is unlikely that the pigment can act as an oxygen carrier, nor is any oxygen liberated from egg extracts by the addition of potassium ferricyanide. Wigglesworth (1942) investigated a haemoprotein occurring in the eggs of the blood-sucking hemipteran *Rhodnius prolixus*, but was unable to demonstrate any function for the pigment. He suggested that such pigments are merely functionless inclusions derived from the blood of the host. However, Lemberg and Legge (1949) have suggested that perhaps haematin compounds in the eggs of blood-sucking insects serve as reserve food for the construction of haematin enzymes before the larvae receive blood. In this connection, it is interesting to note that Riek (personal communication) found that larvae of *B. microplus* feeding on bovines do not ingest red cells, but appear to obtain their nutritional requirements from tissue fluids. Also, Frick (1936), quoted by Schulze (1955), suggested that the pigments of the cuticles of ixodids (reddish brown to very dark brown, more rarely green) are probably derivatives of host blood pigment.

However, extraction of aging eggs and larvae has shown that as the concentration of haemixodovin decreases with time, increasing amounts of a haematin-like compound can be recovered. Concomitantly, increasing amounts of a black pigment are deposited in the larval gut. Therefore, while a small proportion of the egg haematin may be used in the construction of larval haematin enzymes, or in cuticle pigmentation, circumstantial evidence indicates that, in the main, only the protein portion of the haemixodovin molecule is utilized by the larva as a reserve food, the discarded haematin being left as a deposit in the larval gut. None of this deposit is excreted as faeces while the larva remains unfed, but Enigk and Grittner (1952) have described the evacuation of reddish black faeces by ixodid larvae after they had attached to a host and commenced blood sucking. These authors explain this delayed evacuation of faeces on anatomical grounds.

The rapid decline in vitelline reserves of haemoprotein in developing eggs is in accord with the assumption that metabolic activity in the growing embryo would be proceeding rapidly, with consequent rapid utilization of reserves. After emergence of the larvae, followed by hardening of their cuticles for a few days, larval metabolism would probably be reduced to a minimum, the larvae then lying dormant. It is notable that during this resting period only minor changes occurred in the amounts of haemixodovin and haematin extracted from the larvae. The drop in concentration of haemoprotein recorded from larvae between the seventh week and the time that the final extracts were made is not readily explained. As there was an interval of 10 days between these last two extractions, the greatest fall may have occurred during the last day or two before the final extraction.

Hitchcock (1955) has found that, after the first deaths occur in a batch of incubated unfed *Boophilus* larvae, almost all the larvae in the batch die within a few days. Thus the majority of living larvae in the final sample extracted would be virtually moribund, and one would expect larval metabolism to have altered from the optimum. If there was an increased utilization of haemoprotein after the seventh week due perhaps to exhaustion of other vitelline reserves, increased larval haematin deposits would have been expected. No such increase occurred. Possible effects on larval metabolism by the rickettsial-like organisms reported from *B. microplus* larvae by Pierce and Pierce (1956), and seen by the author in the larvae he extracted, also should be borne in mind.

In view of the presence of an unidentified pink compound in alkaline extracts of old larvae, it is possible that haematin is not the sole end-product of haemixodovin breakdown. Figure 3 indicates that there was a slight fall in larval haematin between the sixth and ninth weeks of incubation, and as no haematin was recovered from larval excreta, it may well be that some haematin is catabolized to form the pink unknown. Haemoprotein catabolism in mammals leads to the formation of bile pigments or porphyrins, but the pigment did not display the characteristic four-banded spectrum of porphyrins and showed none of the solubility or reactions characteristic of any of the known bile pigments (Lemberg and Legge 1949). However, this does not preclude the possibility of the unknown being a tetrapyrrole or dipyrrole pigment of a type hitherto undescribed. Whatever the nature of this pigment, the fact remains that at the time of death large deposits of haematin are present in the larval gut, and it would seem that haematin is the main end-point of haemixodovin catabolism in these larvae.

One further set of observations remains to be considered. Hunt and Collins (1896) cite Wynne's observations that *B. microplus* collected from cattle with severe anaemia due to heavy tick infestation have a translucent appearance due to the lack of haemoglobin in their diet. Hitchcock (unpublished data) showed that translucent ticks laid fewer and lighter-coloured eggs than did ticks collected from healthy cattle. Furthermore, fewer larvae hatched from eggs of translucent ticks than from "normal" eggs. Although this lowered fertility of translucent ticks may be due to factors other than a reduced haemoglobin intake, the possibility remains that the presence of a minimum level of protein-bound haematin may be essential for the normal development of the eggs of *B. microplus*.

IV. Acknowledgments

The author is indebted to Dr. J. E. Falk, Division of Plant Industry, C.S.I.R.O., and Mr. J. E. O'Hagan, Red Cross Blood Transfusion Service, for their advice and assistance in the identification of haematin compounds. Mr. R. F. Riek, Division of Animal Health and Production, C.S.I.R.O., kindly advised on the preparation of the manuscript. The histochemical tests for free iron in sections of ticks were carried out by Mr. J. R. Saal.

V. References

Dresel, E. I. B., and Falk, J. E. (1956).—*Biochem. J.* **63**: 72.

Eigenberger, E. (1931).—*J. prakt. Chem.* **130**: 75.

Enigk, K., and Grittner, I. (1952).—*Z. Tropenmed. u. Parasit.* **4**: 77.

Fairley, N. H. (1941).—*Quart. J. Med.* **10**: 115.

Frick, G. (1936).—*Z. Morph. Ökol. Tiere* **31**: 142.

Hitchcock, L. F. (1955).—*Aust. J. Zool.* **3**: 295.

Hunt, J. S., and Collins, W. (1896).—"Report on Tick Fever." pp. 40–2. (Govt. Printer: Brisbane.)

King, E. J. (1948).—*Lancet* **1**: 478.

King, E. J., and Delory, G. E. (1945).—*Biochem. J.* **39**: 111.

Kunkel, H., and Slater, R. (1952).—*Proc. Soc. Exp. Biol., N.Y.* **80**: 42.

Kunkel, H., and Tiselius, A. (1951).—*J. Gen. Physiol.* **35**: 89.

Lees, A. D., and Beament, J. W. L. (1948).—*Quart. J. Micr. Sci.* **89**: 291.

Lemberg, R., and Legge, J. W. (1949).—"Haematin Compounds and Bile Pigments." (Interscience Publishers Inc.: New York.)

Mitchell, P. H. (1946).—"A Textbook of Biochemistry." (McGraw Hill Book Co. Inc.: New York.)

Nicholas, R. E. H. (1951).—*Biochem. J.* **48**: 309.

Pearse, A. G. E. (1953).—"Histochemistry." (Churchill & Co.: London.)

Pierce, A. E., and Pierce, M. H. (1956).—*Aust. Vet. J.* **32**: 144.

Prunty, F. I. G. (1945).—*Biochem. J.* **39**: 446.

Riek, R. F. (1959).—*Aust. J. Agric. Res.* **10**: 604.

Schulze, P. (1955).—*Z. Parasitenk.* **17**: 217.

Stern, A., and Wenderlein, H. (1934).—*Z. physik. Chem.* A **170**: 345.

Warburg, O., and Negelein, E. (1932).—*Biochem. Z.* **244**: 9.

Wigglesworth, V. B. (1942).—*Proc. Roy. Soc.* B **131**: 313.

THE DIGESTION OF WOOL BY A KERATINOLYTIC BACILLUS

By G. S. Molyneux*

[*Manuscript received February 5, 1959*]

Summary

The occurrence, isolation, and characteristics of an aerobic, mesophilic, spore-forming bacillus capable of separating wool and hair into their constituent cuticular and cortical cells are described.

The organism was isolated from the contents of experimentally induced dermoid cysts implanted on the mid-lateral region of a single sheep. Examination of wool samples from these cysts showed the wool to be degraded, as evidenced by the presence of numerous free cortical and cuticular cells. The contents of experimental cysts on other sheep showed no evidence of bacterial contamination 13 weeks after implantation, and wool samples from these cysts were normal.

Disruption of wool fibres occurred *in vitro* and *in vivo*. The evidence suggests that the organism is capable of attacking native wool protein.

In the case of this one sheep a comparison is drawn between the environment necessary for the development of "pink rot" and the intracystic environment of the wool.

It is also suggested that the technique of cyst implantation could be developed for the production of sterile native (untreated) wool and sterile adult skin.

I. Introduction

Native wool is characteristically resistant to attack by enzymes. This unreactivity can be related to "the existence within wool of a compact three-dimensional polymeric structure" (Geiger *et al.* 1941) which is the result of intermolecular disulphide and hydrogen bonds (Crewther 1956).

Of the microorganisms known to degrade wool the majority occur among the dermatophytes such as *Microsporum gypseum* and members of the genus *Trichophyton* (White, Mandels, and Siu 1950; Vanbreuseghem 1952). Keratinolytic enzymes have been described from *Aspergillus flavus* and *Aspergillus niger* (Koh, Santoro, and Messing 1958) and the growth of *Candida albicans* on keratin as sole source of nitrogen has been reported (Kapica and Blank 1957). Aerobic, mesophilic, spore-forming members of the genus *Bacillus* have also been implicated in the degradation of wool. Waters (1932) described an organism resembling *B. vulgatus* which was found to cause "pink rot" of wool, whilst Hirschmann, Zametkin, and Rogers (1944) found that degreased, steam-sterilized wool in the presence of added nutrient supported growth of *B. mesentericus* and *B. subtilis*. Crewther, McQuade, and Pressley (unpublished data) also refer to strains of *B. subtilis* which attack wool.

The purpose of this paper is to report results which confirm and extend some of previously published data and to describe the occurrence, isolation, and characteristics of another aerobic, mesophilic, spore-bearing rod, which is capable of separating wool and hair into their constituent cuticular and cortical cells.

*Institute of Dental Research, United Dental Hospital, Sydney.

II. Isolation of the Bacillus

In order to study the mechanism of cyst growth, a technique was developed for producing standard, experimental, implantation dermoid cysts on the mid-lateral region of sheep. Three Merino and one Corriedale sheep were used, as many as three cysts being implanted on the side of a single animal. Before surgical implantation the skin was prepared by close clipping, defatted with alcohol and ether, then washed with soap and water followed by the application of "Cetavalon".

At intervals after implantation, e.g. after 5, 13, 24, and 52 weeks, the fluid cyst contents were evacuated by syringe and tested for sterility. A standard biopsy specimen was then taken from each cyst together with a sample of intracystic wool.

The aspirated cyst fluids were examined for the presence of bacteria by means of unstained and stained preparations, and by culturing aerobically and anaerobically on sheep blood agar and in Robertson's anaerobic cooked meat medium. As was to be expected, at 5 weeks all cysts were contaminated. In tubes of the cooked meat medium inoculated from two cysts from the one sheep and incubated anaerobically, proteolysis was very obvious. At 13 weeks, no evidence of bacterial contamination was shown by any cyst other than those the contents of which were previously proteolytic for meat. These cyst contents again caused rapid proteolysis.

Serial dilutions of the supernatant from an anaerobic meat culture were made, sown on blood agar, and incubated under aerobic and anaerobic conditions. The resulting culture was mixed and contained:

(i) An aerobic, Gram+, spore-forming, motile rod.
(ii) A strictly anaerobic, Gram+, non-sporing, motile rod.

Heavy growth of organisms occurred at 26 and 37°C. The colonies of the aerobic organism had a characteristically heaped, mucoid appearance; those of the anaerobic organism were flat and rhizoid.

Because these organisms in mixed culture were strongly proteolytic for meat and were recovered from an environment containing wool, it was decided to examine the effect of incubating the organisms with hair. Samples of black human hair were washed with petroleum ether for 15 min, dried at room temperature, washed in distilled water, placed in 20 ml nutrient broth, and autoclaved at 120°C for 30 min. The medium was then inoculated from an anaerobic meat culture. On attempting to remove the hair 14 days later it was found to be brittle and fractured, although macroscopically it seemed normal. Microscopically it appeared normal along its length but was frayed at the ends; on applying gentle pressure on the coverslip the hair disintegrated and numerous, free, spindle-shaped cortical cells and flattened cuticular cells could be distinguished. In this experiment there was no surface pellicle formation and the pH value of the culture was 7·2.

At this stage it was considered that enzymic digestion of an intercellular matrix was occurring. To study this possibility and to ascertain whether the organisms responsible could utilize nitrogen from keratin alone, human hair and Merino wool were placed in separate tubes containing Kisch solution (a mineral medium free of nitrogen (Kisch 1919)), autoclaved at 120°C for 30 min, and inoculated from the broth–hair culture. Growth occurred in both hair and wool tubes and was accompanied by disruption of the fibre. This was much more evident for wool than for hair and was characterized by lifting of the cuticle from the cortex to form empty envelopes of cuticular cells and free cortical cells. An offensive odour, similar to that of fatty acids, noticed in anaerobic meat and broth cultures was absent from Kisch-solution cultures.

At this point subcultures prepared from salts–wool cultures showed that the anaerobic rod had not survived and that the aerobic rod had thrown two variants: rough and mucoid. Therefore it was decided to investigate the aerobic organism.

On successive transfers in mineral medium growth improved and pigmentation was noticed; wool fibres near the surface of the fluid were coloured pink and showed large numbers of adhering organisms.

III. Classification of the Organism

In order to identify this microorganism among the aerobic, mesophilic, spore-forming bacteria, reference was made to the classification and biochemical reactions used by Smith, Gordon, and Clark (1946). Eight strains, distinguished by slight variation in colony form, were selected from broth and salts cultures for examination. As the parent strain a mucoid colony was selected and a rough colony type as a variant. In successive transfers the latter seemed to be the more stable.

The following characteristics were observed:

Vegetative rods.—There was great variation in morphology, rods $0\cdot5$–$1\cdot0\ \mu$ by 1–5 μ being seen; arranged as single cells or in chains up to 50 μ; ends rounded; uniform or granular staining of cytoplasm; Gram+; motile in an 18-hr nutrient broth culture, but non-motile and spore-forming in mineral medium.

Sporangia.—No swelling by spores.

Spores.—1 by $1\cdot5\ \mu$, oval, central, or subterminal.

Growth.—Occurred at 26 and 37°C.

Colonies.—Mucoid type: large, raised, entire, smooth, spreading—this is considered to be the parent type of colony. Rough type: small, raised, granular, entire, or lobate, sunk in medium.

Nutrient agar slant.—Growth fair to abundant, opaque to thin and spreading.

Glucose nutrient agar slant.—Growth more abundant and softer than on nutrient agar. Cream to pink pigmentation.

Potato.—Abundant growth, slimy; potato darkened; pink pigmentation.

Nutrient broth.—Moderate growth, uniform turbidity, slight sediment with some strains. Light to heavy, tough, wrinkled, surface pellicle. On standing, mucous droplets sometimes formed on surface of pellicle. Pink pigmentation sometimes on undersurface of pellicle.

Physiological reactions.—Fermentation reactions resulted in the production of acid without gas from glucose, maltose, sucrose, fructose, mannitol, arabinose, mannose, salicin, dextrin; variable reactions with inulin and glycerol; no reaction with lactose, galactose, raffinose, rhamnose, and xylose.

Acetylmethylcarbinol was produced as judged by the Voges-Proskauer reaction, citrate was utilized for growth, and hydrolysis of the following substances occurred:

starch, gelatin (10 days), casein, egg yolk (7 days), egg white, and coagulated serum. Neither indole nor hydrogen sulphide was produced and nitrate was not reduced to nitrite. Methylene blue was reduced and was not reoxidized in 21 days.

The above reactions were obtained with seven of the selected strains; one strain, however, differed in giving a rough granular growth on potato, poor growth in citrate, and although it initially reduced methylene blue, this was reoxidized in 5 days. In wool–salts medium this strain failed to produce pink pigmentation, and splitting of the fibres was negligible.

From the above characteristics it would appear that the organism belongs to group I of the genus *Bacillus* (Smith, Gordon, and Clark 1946). It resembles the organism that Waters (1932) associated with "pink rot" and probably is a variant with characteristics between those of *B. subtilis* and *B. pumilus*.

IV. Effect of the Organism on Wool

To correlate the above biochemical reactions with the ability to degrade wool fibres, eight tubes each containing Kisch solution and Merino wool fibres were inoculated with one of the eight colony types and incubated at 37°C. The fibres had first been washed for 15 min in petroleum ether at room temperature, washed in distilled water, then placed in 10 ml Kisch solution and sterilized by autoclaving at 120°C for 30 min. The cultures were examined after 14 days. Deep pink pigmentation was found to be characteristic of degradation of Merino wool. In a similar experiment other keratinous fibres were used as a nitrogen source. There was slight pigmentation with Corriedale wool and no pigmentation with bandicoot (*Perameles nasuta*) and human hair.

The pigmented material lay on the surface of the salts solution as a light surface growth and was concentrated on the fibres near the surface. Unstained preparations showed numerous organisms, often in long filaments, surrounding the fibres. The completeness of the degradation, as judged by the presence of free cortical cells, is positively correlated with the intensity of pigmentation. As previously mentioned, one of the strains selected for examination did not produce pigment or extensive degradation of wool.

Fibres (mounted in water), which appeared to be normal and could be removed intact from the salts solution, could be disintegrated by applying *gentle* pressure to the coverslip (Plate 1, Fig. 1). The cuticular cells, separated from the cortical cells, were observed as empty envelopes or as fragments of a few overlapping flattened scales. Spindle-shaped cortical cells were seen clearly, either separated within the cuticular sheath or as free cells (Plate 1, Fig. 2).

At 14 days the appearance was indicative of selective removal of an intercellular matrix, as in the early enzymic digestion of reduced wool by trypsin. At 56 days the amount of wool seemed to have diminished and to consist of short lengths and free cells in a compact mass at the bottom of the tube (Plate 2, Fig. 1). When such fragments were placed on a slide and gentle pressure applied to the coverslip, individual cortical cells could be disrupted or teased apart to reveal an intracellular. fibrillar structure, indicating the removal or breakdown of an intracellular matrix

(Plate 2, Fig. 2). Prior to application of pressure small fragments resembling fractured fibrils were seen, probably indicating eventual digestion of keratin. These observations are in accord with those of Hock, Ramsay, and Harris (1941) on the enzymic digestion of reduced wool.

Because wool fibres previously soaked in an alkaline medium are susceptible to enzymic attack (Crewther 1956), the pH value of the salts medium was determined during the course of the observations. On preparation, the pH value of Kisch solution was 7·3; of Kisch solution and wool after autoclaving at 120°C for 30 minutes, 6·8; after 14 days incubation, 7·5–7·9; after 56 days, 6·8 (control at 56 days, 6·4); after 84 days, 6·1.

Mercer (1953) showed that wool fibres cut into short lengths and heated at 120°C for $1\frac{1}{2}$ hr underwent supercontraction, after which the ortho-cortex was more susceptible to enzymic digestion. Crewther (1956) also showed that wool autoclaved in water for 1 hr at 120°C was susceptible to tryptic attack.

In order that the breakdown of wool could be evaluated, wool fibres prepared as before were placed in Kisch solution and autoclaved at 120°C for 30 min. These fibres were susceptible to attack by crystalline trypsin (Armour) and were then easily fragmented and free cortical cells were seen. When fibres were not autoclaved they were resistant to enzymic attack. Thus, although some method of sterilizing the wool fibre is necessary before the effect of the organism in pure culture can be examined, autoclaving at 120°C for 30 min, a procedure necessary to sterilize wool when contaminated with spores, increases the susceptibility of the fibre to enzymic digestion.

A further experiment was planned to show the effect of the organism on defatted, unsterilized wool. A sample of Merino wool was defatted by soaking in petroleum ether for 10 min, then allowed to dry in air at room temperature. The defatted wool fibres were placed in a 40-day culture of the organism containing autoclaved Merino wool in Kisch solution. The culture (suspension of organisms) was transferred to another tube, care being taken not to disturb the degraded and fragmented wool fibres at the bottom of the tube. The defatted, unsterilized wool fibres were then placed in this culture, of pH value 7·3. Ten days later wool fibres were removed and, on microscopic examination, cuticle cells were seen to be lifting from the partially fractured fibres. After 14 days disruption of the fibres had proceeded further and when gentle pressure was applied free cortical cells were seen, as in Plate 1, Figure 1.

To determine if contaminants had been introduced with the defatted, unsterilized wool the culture at 14 days was tested on blood agar. Mucoid, rough, and smooth colonies were observed and stained preparations from each showed the colonies to be composed of Gram+ rods. If a contaminant had been introduced into the pure culture it had not survived or it was indistinguishable from the test organism.

V. Discussion

An aerobic, mesophilic, spore-forming rod isolated from experimental cyst contents has been shown to utilize degreased autoclaved wool as a sole source of nitrogen. Water-soluble material such as urea and uric acid (Bolliger 1951; Bolliger and Gross 1952) extracted from wool fibres during autoclaving could be expected to provide the nitrogen source necessary for initial growth. However, as Crewther (1956) has emphasized, this does not mean that the organism will attack native wool protein, since it has been demonstrated that sterilization (autoclaving at 120°C for 30 min), "disorders" the protein sufficiently to allow digestion by trypsin.

Evidence is presented, however, that this organism *can* attack native wool protein. Defatted, unsterilized wool was degraded in a manner similar to that described for autoclaved wool. Samples of intracystic wool from all experimental sheep were examined. Degraded wool, as evidenced by the presence of free cortical cells, was found only in cysts of one Merino sheep; these cysts had given evidence of contamination on all occasions when examined. The cyst contents from the other animals gave no evidence of contamination at 13 weeks. Thus it would appear that the keratinolytic organism was in this instance able to adapt itself successfully to the new environment.

The intracystic environment has not been investigated except for periodic determinations of the pH value of cyst contents.* Crewther (1956) showed that the soaking of wool (extracted with petroleum ether and ethanol at room temperature) in buffers influenced the susceptibility to tryptic attack but that at 40°C there was evidence of digestion only when the pH of the pretreatment buffer exceeded the value of 9. As yet there is no evidence that intracystic wool would be disordered by continued soaking in cyst fluids which are presumed to be a combination of suint and tissue exudate.

Koh, Santoro, and Messing (1958) reported the elaboration of keratinolytic enzymes by *Aspergillus flavus* and *A. niger*, as judged by their effect on partially degraded wool, hoof meal, or defatted wool as the sole source of carbon and nitrogen. They found that keratinolytic enzymes were not produced in the presence of chicken feathers or defatted human hair as substrate or when the organisms were grown in broth.

In this study it was also found that the nature of the substrate and medium influenced the ability of the organism to degrade keratinous fibres. When hair or wool *broth* media were inoculated, a heavy, surface pellicle usually developed as in nutrient broth, but disruption of the fibres occurred only in old cultures. With salts cultures containing hair or wool, surface growth was scant but disruption occurred more rapidly. These observations suggest that the organisms preferentially utilize the more readily available nitrogen source in nutrient broth and attack hair or wool only when this nitrogen source has been depleted. In all cases wool was attacked more rapidly than human hair.

An interesting aspect of the metabolism of this organism is the production of pink pigment, which is best developed in salts–Merino wool medium. This pigmentation is of particular interest when the aetiology of fleece rot is considered. Fleece rot ("water rot" as described by Seddon (1931)) is an abnormality of the fleece of sheep which follows prolonged exposure to rain. It is characterized by superficial dermatitis and a serous exudate which on drying causes matting of the wool fibres (Hayman 1953). The affected wool becomes hard and stringy and in severe cases may be easily plucked from abnormal areas of skin. Pigmentation of the wool may or may not also be associated with fleece rot.

---

*The pH of the contents of a cyst containing disrupted wool measured at 8, 13, 24, and 36 weeks ranged from 6·8 to 8·0.

Chromogenic bacteria have been shown by Seddon (1937) to be the cause of the coloration associated with fleece rot and these colorations either persist in the fibres or can be scoured out. However, Waters (1932) described a condition he termed "pink rot", in which the wool was pigmented and *underwent disintegration.* The causative organism was an aerobic, mesophilic, spore-forming rod, and was capable of breaking wool into individual cells *in vitro.*

Hayman (1953), in producing fleece rot experimentally, mentions a pink coloration and rotting which developed after incubating moist wool for 3 weeks. No mention is made of a causative organism.

In the present study it would appear that one of the animals in the series carries a keratinolytic organism; this organism was isolated from experimental implantation cysts and is described herein. The technique of cyst implantation in this instance permits the reproduction of the environmental conditions necessary for the development of pink rot—wool exposed for a long period to bacteria in a fluid medium (suint and inflammatory exudate).

Since the cyst contents of the other animals in the series gave no evidence of bacterial contamination 13 weeks after implantation and the intracystic wool appeared to be normal, there is every indication that this technique of cyst implantation could be developed, possibly in conjunction with the use of antibiotics, to provide a source of sterile native (i.e. untreated) wool. Such a technique would also be valuable in providing sterile adult skin for tissue culture.

VI. Acknowledgments

The author is indebted to Dr. N. E. Goldsworthy, Director, Institute of Dental Research, for advice and guidance during this work, and to Mr. H. C. Spies, Institute of Dental Research, for assistance in the classification of the organism.

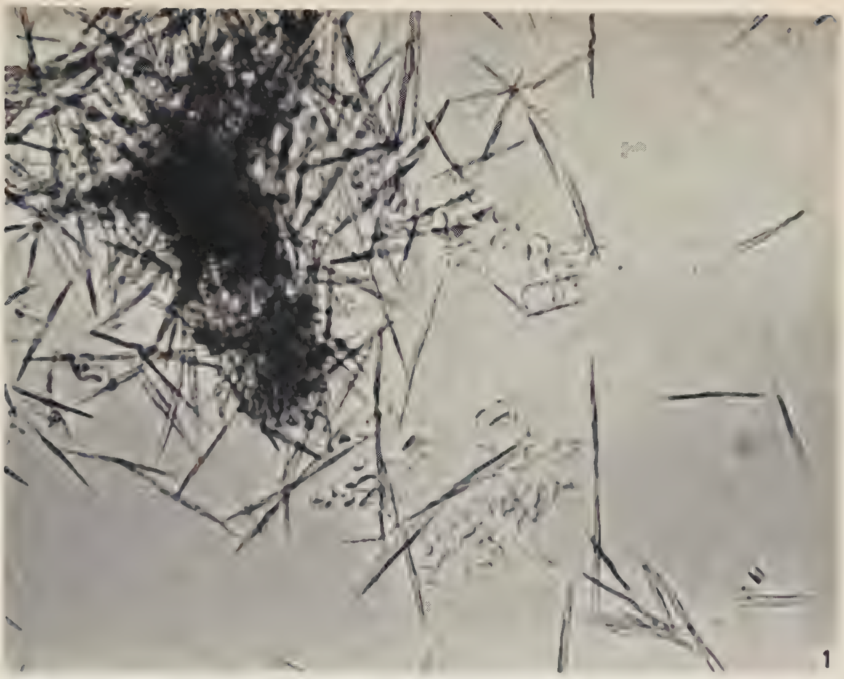
Through the courtesy of Dr. D. F. Stewart, Officer-in-Charge, McMaster Animal Health Laboratory, C.S.I.R.O., Glebe, N.S.W., and Dr. I. W. McDonald, Officer-in-Charge, Sheep Biology Laboratory, C.S.I.R.O., Prospect, N.S.W., facilities and animals were made available for the experimental work.

Dr. H. R. Sullivan, Assistant Director, Institute of Dental Research, Professor K. W. Cleland, University of Sydney, and Mr. P. G. Schinckel, Dr. A. G. Lyne, and Dr. B. F. Short, Division of Animal Health and Production, C.S.I.R.O., kindly read the manuscript. Dr. A. G. Lyne and Dr. B. F. Short provided samples of bandicoot hair, Merino, and Corriedale wool.

Also I wish to thank Mr. B. Rumble, United Dental Hospital of Sydney, for assistance in the preparation of the photomicrographs.

The author is also indebted to the National Health and Medical Research Council for a grant to enable this work to be carried out.

BACTERIAL DIGESTION OF WOOL



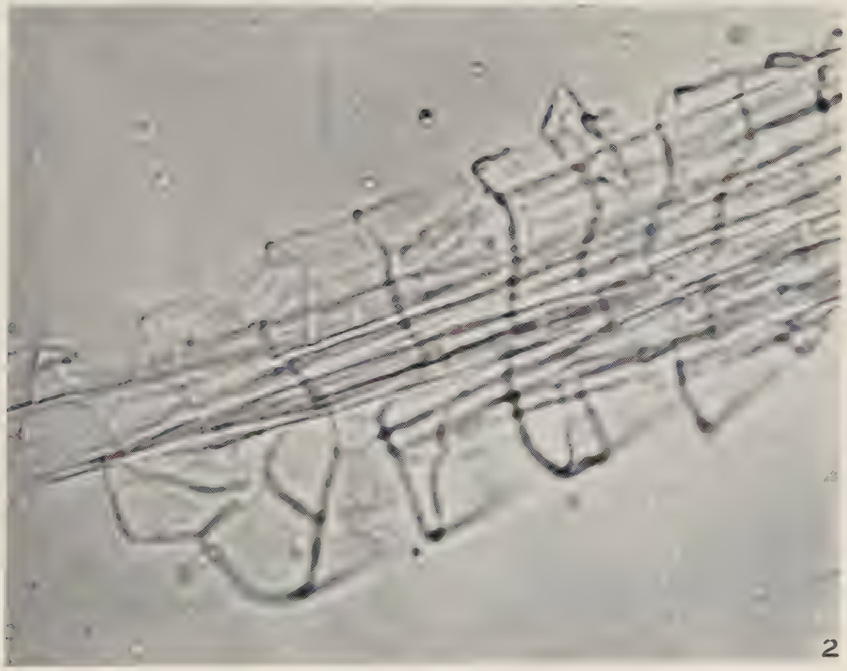
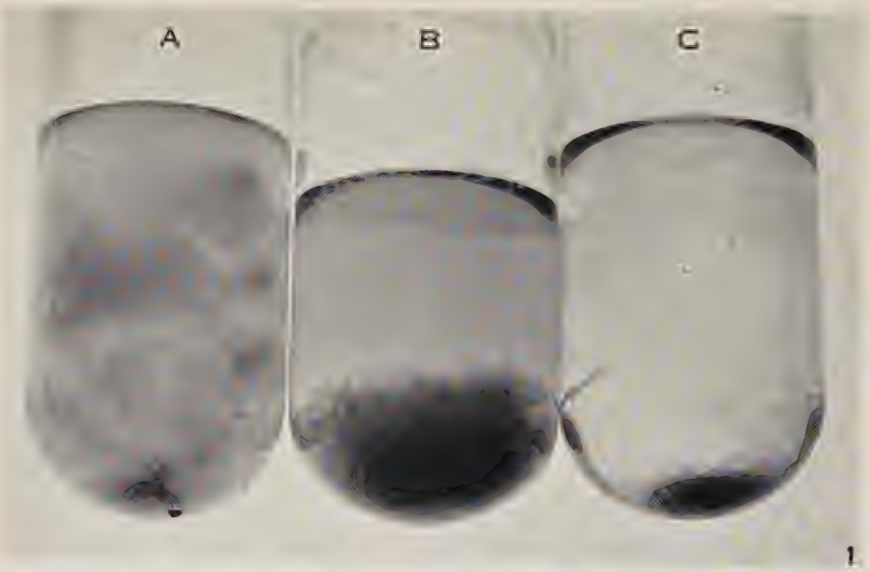


Fig. 1.—Photomicrograph of Merino wool fibres, taken from a 14 days wool salts culture, showing disruption of fibres, after applying gentle pressure, with release of free cortical and cuticular cells.

Fig. 2.—Photomicrograph of Merino wool fibre showing cortical cells lying loosely within cuticular sheath.



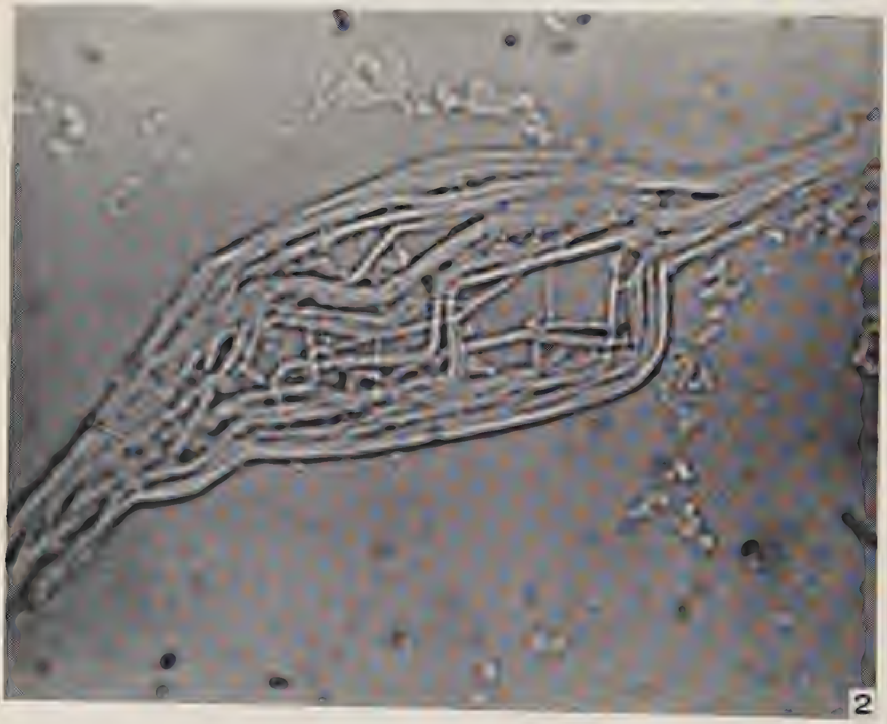


Fig. 1.—Tubes of wool-salts culture showing dissolution of wool: *A*, control tube (uninoculated); *B*, after 56 days incubation; *C*, after 84 days incubation.

Fig. 2.—Photomicrograph of single cortical cell showing intracellular fibrils.

VII. REFERENCES

BOLLIGER, A. (1951).—Water extractable constituents of hair. *J. Invest. Derm.* **17**: 79–84.

BOLLIGER, A., and GROSS, R. (1952).—Nitrogenous compounds in the aqueous extract of vertebrate keratinous structures. *Aust. J. Exp. Biol. Med. Sci.* **30**: 399–408.

CREWTHER, W. G. (1956).—Pretreatments which affect the susceptibility of wool to proteolysis. I. The effects of pH, wetting agents and solvent extraction. Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. C. pp. C-227–56.

GEIGER, W. B., PATTERSON, W. I., MIZELL, L. R., and HARRIS, M. (1941).—Nature of the resistance of wool to digestion by enzymes. *Bur. Stand. J. Res., Wash.* **27**: 459–68.

HAYMAN, R. H. (1953).—Studies in fleece-rot in sheep. *Aust. J. Agric. Res.* **4**: 430–68.

HIRSCHMANN, D. J., ZAMETKIN, M., and ROGERS, R. E. (1944).—The utilization of wool by four saprophytic micro-organisms in the presence of additional nutrients. *Amer. Dyest. Rep.* **33**: 353–9.

HOCK, C. W., RAMSAY, R. C., and HARRIS, M. (1941).—Microscopic structure of the wool fibre. *Bur. Stand. J. Res., Wash.* **27**: 181–90.

KAPICA, L., and BLANK, F. (1957).—Growth of *Candida albicans* on keratin as sole source of nitrogen. *Dermatologica* **115**: 81–105.

KISCH, B. (1919).—Die Verwertbarkeit verschiedener chemischer Verbindungen als Stickstoffnahrung für einige pathogene Bakterien. *Zbl. Bakt.* (*I Abt. Orig.*) **82**(1): 28.

KOH, W. Y., SANTORO, A. F., and MESSING, R. A. (1958).—Keratinolytic enzymes from *Aspergillus flavus* and *Aspergillus niger*. *Bact. Proc.* A**38**: 18.

MERCER, E. H. (1953).—The heterogenicity of the keratin fibres. *Text. Res. J.* **23**: 388–97.

SEDDON, H. R. (1931).—Conditions which predispose sheep to blowfly attack. *Agric. Gaz. N.S.W.* **42**: 581–94.

SEDDON, H. R. (1937).—Bacterial coloration of wool in studies in the sheep blowfly problem. Sci. Bull. Dep. Agric. N.S.W. No. 54.

SMITH, N. R., GORDON, R. E., and CLARK, F. E. (1946).—Aerobic, mesophilic, spore-forming bacteria. Misc. Publ. U.S. Dep. Agric. No. 559.

VANBREUSEGHEM, R. (1952).—Keratin digestion by dermatophytes: a specific diagnostic method. *Mycologia* **44**: 176–82.

WATERS, R. (1932).—Pink rot of wool. *N.Z. J. Agric.* **44**: 35–8.

WHITE, W. L., MANDELS, G. R., and SIU, R. G. H. (1950).—Fungi in relation to the degradation of woollen fabrics. *Mycologia* **42**: 199–223.

STUDIES ON OXIDIZED WOOL

I. A COMPARISON OF THE COMPLETENESS OF OXIDATION WITH PERACETIC AND PERFORMIC ACIDS

By E. O. P. Thompson* and I. J. O'Donnell*

[*Manuscript received April 1, 1959*]

Summary

The oxidation of wool with performic and peracetic acids has been compared by measuring the cysteic acid contents of hydrolysates of the oxidized wool. Whereas virtually complete oxidation takes place with performic acid, the oxidation with peracetic acid is incomplete. Performic acid can also further oxidize the partial oxidation products in wool treated with peracetic acid and it is concluded that performic acid is the more powerful oxidizing agent for wool. For the oxidation of the disulphide bonds prior to the extraction of proteins from wool, performic acid is therefore the better reagent.

I. Introduction

Performic acid has been extensively used for the oxidation of disulphide linkages in proteins following its successful application by Sanger (1949*a*) to the separation of the two peptide chains of insulin. From the results obtained with insulin (Sanger 1949*b*), lysozyme (Jollès-Thaureaux, Jollès, and Fromageot 1958; Thompson 1958), ribonuclease (Hirs 1956), papain (Kimmel, Thompson, and Smith 1955), chymotrypsin (Meedom 1956), and other proteins it is known that no splitting of peptide bonds takes place. Apart from the oxidation of cysteine and cystine residues to β-sulphoalanyl (cysteic acid) residues, the only other amino acids which are modified by the reagent are methionine and tryptophan, provided precautions are taken to avoid chlorination of tyrosine residues (Thompson 1954, Hirs 1956). Methionine is converted to the sulphone (Hirs 1956) and tryptophan takes up three atoms of oxygen (Toennies and Homiller 1942) and is converted to a product of unknown structure.

Aqueous peracetic acid was used for the oxidation of wool by Alexander, Hudson, and Fox (1950) because it is stable in water, has a specificity similar to performic acid, and dilute solutions dissolve very little wool. Performic acid is unstable; it is decomposed by water and in the presence of the large amount of formic acid necessary for its preparation considerable amounts of oxidized wool dissolve. For commercial treatment of wool fibres peracetic acid is therefore preferable. As a preliminary step in the isolation of wool proteins, however, this is not the case, since the results presented in this paper show that oxidation with peracetic acid is incomplete, whereas after performic acid oxidation virtually all the cysteine and cystine of wool may be detected as cysteic acid in hydrolysates of the oxidized wool.

---

* Division of Protein Chemistry (formerly Biochemistry Unit), C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

Alexander, Hudson, and Fox (1950) and Corfield, Robson, and Skinner (1958) studied the completeness of oxidation with peracetic acid by measuring the cystine content of hydrolysates after various periods of oxidation. This is an indirect method which can give erroneous results in that cystine may be oxidized to partial oxidation products which no longer analyse as cystine but which may be capable of further oxidation to β-sulphoalanyl residues.

In our experiments we have preferred analyses for cysteic acid in hydrolysates of oxidized wool for a true assessment of the completeness of oxidation.

As a preliminary step in the isolation of proteins from oxidized wool (O'Donnell and Thompson 1959) complete oxidation of the modified amino acid residues is desirable for complete splitting of disulphide cross-linkages and the formation of a stable derivative from cysteine and cystine residues.

II. Materials and Methods

Merino 64's wool in the form of solvent-degreased top was used. To remove residual oil the top was extracted four times with light petroleum, then with ethanol, and repeatedly with distilled water before being dried and conditioned at 63 per cent. R.H. and 20°C. The nitrogen (Kjeldahl) and sulphur (Zimmerman) contents of the dry wool were 16·9 and 3·26 per cent. respectively. Moisture contents were determined by overnight drying in an oven at 105°C. The ash content (800°C) was 0·16 per cent.

Performic acid reagent was prepared by mixing 1 vol. 30 per cent. hydrogen peroxide and 9 vol. 98–100 per cent. formic acid and allowing to stand 1–2 hr at room temperature before use (Toennies and Homiller 1942).

Peracetic acid (Laporte Chemicals) contained sulphuric acid catalyst. Titration by the methods of Swern (1953) showed the reagent to be 40 per cent. (w/v) peracetic acid with 6 per cent. hydrogen peroxide but no diacetyl peroxide was present.

III. Experimental

(a) *Oxidation with Performic Acid*

Samples of wool (approximately 30 mg), equilibrated at 63 per cent. R.H. and 20°C, were cooled to 0°C in "Quickfit" stoppered test tubes. Performic acid reagent (1 ml) cooled to 0°C was added (i.e. at least 10 times the theoretical amount required to oxidize the known cysteine, cystine, methionine, and tryptophan content) and reaction continued at 0°C for 1–48 hr. Ice-cold water (10 ml) was then added and the mixture freeze-dried. The residue was hydrolysed under reflux with 2 ml 6N HCl (which had been twice distilled in glass) in an oil-bath at 138–140°C for 24 hr. The hydrolysate was diluted to 25 ml and 1-ml samples freeze-dried before analysis.

(b) *Oxidation with Peracetic Acid*

Peracetic acid (40 per cent. w/v) was diluted with the required solvent to give 1·6 per cent. or 5 per cent. peracetic acid (Alexander, Hudson, and Fox 1950). These authors studied only aqueous solutions of peracetic acid in which more hydrogen peroxide is formed on dilution due to hydrolysis of peracetic acid until equilibrium is attained. We have also studied dilute solutions of peracetic acid in acetic acid and formic acid where equilibrium lies in the direction of less hydrogen peroxide.

In all experiments the liquor : wool ratio was 50 : 1.

Analytical small-scale experiments were carried out as described for performic acid. The residual fibres from larger-scale oxidations were analysed after washing the oxidized fibre free from reagent, drying, and conditioning. The oxidized fibres (approximately 40 mg) were hydrolysed under reflux as described above.

(c) *Analyses for Cysteic Acid*

Cysteic acid was separated from the other amino acids on 20-cm columns of sulphonated polystyrene resin ("Dowex-50 X8") equilibrated with 0·2N sodium citrate buffer at pH 3·1 (Moore and Stein 1954). The pH 3·1 buffer is preferable to the pH 3·42 buffer previously used (Kimmel, Thompson, and Smith 1955; Thompson 1956) since it gives a wider separation between cysteic acid and aspartic acid.

The accuracy of the analyses was improved by using an internal standard. The standard cysteic acid was passed through the column (3 ml of a 0·3 mM solution followed by 2 × 0·5 ml) and then 8 ml buffer. The freeze-dried sample was dissolved in buffer (usually 4 ml) and then 3 ml was added to the column, washed in with buffer (2×0·5 ml) and eluted with more buffer. A total of 35 tubes, containing equal-sized fractions of approximately 1 ml, were collected. To each tube was added one drop of 3·5N NaOH to bring the pH to 5, the tubes shaken, and analysed with 2 ml ninhydrin reagent according to the method of Moore and Stein (1948). By adding the cysteic acid sample in 3 ml buffer, the peak was spread over five tubes and enabled more accurate measurement and integration of the optical densities. Optical densities were measured in a Coleman Junior spectrophotometer at 570 mμ.

IV. RESULTS

(a) *Sulphur Balance on Wool Samples*

The total sulphur of the wool top was 3·26 per cent. This is equivalent to 1019 μg-atoms S/g. Allowing 40 μg-atoms S/g for the methionine content of wool (Simmonds 1956), the total –S–S– and –SH groups of wool should contribute 979 μg-atoms S/g, assuming there are no unknown sulphur-containing compounds in wool. On analysis of a sample of wool top by polarographic methods on the intact fibre (Leach 1959), 465 ± 15 (95 per cent. confidence limits) μmoles cystine/g and 39 ± 3·3 (95 per cent. confidence limits) μmoles cysteine/g were found. This is equivalent to 969 ± 30 (95 per cent. confidence limits) μg-atoms S/g, in excellent agreement with the cysteine plus cystine content calculated from the total sulphur analysis.

Complete oxidation of the cysteine plus cystine of this wool should therefore give 969–979 μmoles cysteic acid/g.

(b) *Oxidation with Performic Acid*

Analyses at different times of oxidation showed that in 1 hr at 0°C 90 per cent. of the maximum yield of cysteic acid was obtained and that, after 4 hr, oxidation was virtually complete. The precision of the analyses was not sufficient to detect any significant difference between the yields of cysteic acid after oxidation at 0°C for 6 hr, 16 hr, or longer. The shorter time of oxidation would reduce the possibility of secondary changes in other amino acid residues or peptide bonds. However, polarographic analyses (Leach 1959) have shown that although no cystine is detectable in the intact fibres in either case, there was 16–20 μmoles/g cystine in hydrolysates of wool oxidized for 5–6 hr but none in the hydrolysates of samples oxidized for 21 hr (see Table 6). For this reason we have used overnight periods (i.e. at least 16 hr) of oxidation before fractionation of the oxidized wool as described in Part II of the series (O'Donnell and Thompson 1959).

Table 1

CYSTEIC ACID IN HYDROLYSATES OF WOOL OXIDIZED WITH PERFORMIC ACID AT 0°C

Time of Oxidation (hr)	Cysteic Acid Content* (μmoles/g wool)
7	971
24	938, 953
41	958, 989

* Each value is the result of a single analysis for a separate oxidation.

In Table 1 the results of cysteic acid determinations on samples of wool oxidized with performic acid are shown. The mean value of these analyses, and those for 24-hr periods of oxidation or longer with peracetic acid in formic acid (see Table 4) where oxidation is complete, is 965 ± 18 (95 per cent. confidence limits). This value, which has not been corrected for any losses during the oxidation, is in excellent agreement with the values calculated above from the sulphur content and the polarographic analyses for cysteine plus cystine. It is of interest that performic acid oxidation of cysteine, cystine, and other proteins (Schram, Moore, and Bigwood 1954; Schroeder *et al.* 1955; Hirs 1956; Hommes, Santema-Drinkwaard, and Huisman 1956; Thompson 1956; Wilcox, Cohen, and Tan 1957) has not always given quantitative conversion of cysteine plus cystine to cysteic acid and most authors have applied a correction factor to allow for this. However, it appears likely that recoveries of cysteic acid will vary with the technique of oxidation and the protein studied. For example, Bidmead and Ley (1958) have obtained yields of 96–103 per cent. of cysteic acid in the oxidation of cystine, and Li (1957) and Jirgensons and Ikenaka (1958) found all the –S–S– bonds in prolactin and human plasma albumin respectively were converted to cysteic acid residues.

With performic acid oxidation it is difficult to measure the weight of the freeze-dried product accurately. Freeze-drying is necessary because of the large amounts of protein going into solution. Following a suggestion by Dr. J. M. Swan, it was found that if the performic acid reagent was saturated with sodium formate the solubility of the oxidized wool was repressed. Whereas 40 per cent. of the wool dissolves during oxidation for 24 hr at 0°C it was found that in the presence of sodium formate, less wool dissolved (weight increase 18 per cent. of the theoretical increase for full oxidation). The cysteic acid content of this residual oxidized wool, after washing with water, was 760 μmoles/g, considerably less than the values obtained in the absence of sodium formate (see Table 1). Hence oxidation has either been incomplete in the presence of sodium formate, or material rich in cysteic acid has dissolved during the isolation of the oxidized fibre.

Table 2

CHANGES IN WEIGHT FOR WOOL OXIDIZED WITH PERACETIC ACID

All oxidations carried out at 28°C except where indicated

Strength of Peracetic Acid and Solvent	Time of Oxidation (hr)	(A) Change in Dry Weight after Oxidation (%)	(B) Material* Dissolved during Isolation (%)	(A + B) Percentage Increase in Weight
1·6 per cent. in water	24 (room temp.)	+3·5	1·1	4·6
1·6 per cent. in acetic acid	24 (room temp.)	+4·2	—	—
5 per cent. in water	8	+2·8	1·5†	4·3
	24	−4·3	7·5†	3·2
	48	−10·0	13·0†	3·0
5 per cent. in acetic acid	8	+3·3	0·5†	3·8
	24	+2·4	1·4†	3·8
	48	+0·7	3·2†	3·9

* Estimated by Kjeldahl nitrogen determination assuming total nitrogen of dissolved material was the same as that of wool.

† It was not appreciated that loss of material may occur during washing with water and this was not measured. For 1·6 per cent. aqueous peracetic acid the losses were 0·8 per cent. in oxidation medium and 0·3 per cent. in washings.

(c) *Oxidation with Peracetic Acid*

The changes in weight of samples of wool oxidized in peracetic acid solutions are shown in Table 2. The theoretical increase in weight, assuming a weight increase of 48 (3 atoms oxygen) for each tryptophan and cysteine residue, 98 (2 × O_3H) for each cystine residue, and 32 (2 atoms oxygen) for each methionine residue, is 5·1 per cent. as shown in Table 3. The tryptophan content of wool is uncertain (Corfield and Robson 1955; Simmonds 1956) but its contribution to the weight increase is small compared to the total of the other amino acids oxidized.

In no case was the theoretical weight increase observed, even after allowing for the material dissolved during isolation of the oxidized fibre. The increase in weight (Table 2) ranged from 60 to 91 per cent. of the theoretical value and was generally less than that expected from the cysteic acid contents given in Table 4. However, the assumption that the nitrogen content of the dissolved material was the same as that of wool may not be justified. Moreover, it was not appreciated that loss of material may occur during washing with water and this was not measured in most cases.

Table 3

CALCULATED INCREASE IN WEIGHT OF WOOL ON OXIDATION

Amino Acid	(A) Amount Oxidized (μmoles/g wool)	(B) Increase in Residue Weight on Oxidation (g)	Increase in Weight (g) for 100 g Wool ($A \times B \times 10^{-4}$)
Methionine	40*	32	0·13
Tryptophan	48†	48	0·23
Cysteine	39	48	0·19
Cystine	465	98	4·56
			Total 5·11

* Simmonds (1956). † Corfield and Robson (1955).

It is of interest to note that the moisture content of the oxidized wools in Table 4 was about 10·6 per cent. which is considerably less than that of the original wool, 12·4 per cent. after equilibration at 63 per cent. R. H. The increase in the number of charged groups ($-SO_3^-$) has not given an expected increase in water uptake.

Table 4 shows the cysteic acid contents of hydrolysates of the freeze-dried reaction mixtures from peracetic acid oxidations of wool, i.e. the whole of the oxidized wool was analysed. The residual oxidized wools, i.e. the washed fibres, have usually increased in weight during oxidation (Table 2) due to uptake of oxygen, but some material has been lost by solution in the oxidizing solution or wash water. The cysteic acid contents of these residual oxidized wools are given in Table 5.

With 1·6 per cent. aqueous peracetic acid, 0·8 per cent. of the total nitrogen of a wool sample was lost in the oxidizing solution and 0·3 per cent. was subsequently washed out by water. The yield of cysteic acid in the freeze-dried reaction mixture (Table 4) averages 88 per cent. of that obtainable with performic acid, and the residual fibre contains 75 per cent. (Table 5).

Our average value of 732 μmoles/g for the cysteic acid content of the residual oxidized fibre obtained with 1·6 per cent. aqueous peracetic acid is in excellent agreement with that of 729 μmoles/g calculated from the data of Corfield, Robson, and Skinner (1958) who also used aqueous peracetic acid. These workers did not consider their value low since it corresponded well with the chromatographic estimates of cystine in their sample of wool. However, their chromatographic cystine value was low compared with that which they obtained by the Shinohara (1935) method. Using their Shinohara value their cystine value is equivalent to 1002 μmoles cysteic acid/g wool. This value would have given sufficient sulphur to account for all the sulphur in their sample apart from the methionine sulphur.

TABLE 4

CYSTEIC ACID IN HYDROLYSATES OF WOOL OXIDIZED WITH PERACETIC ACID

All oxidations carried out at room temperature except where indicated

Strength of Peracetic Acid and Solvent	Time of Oxidation (hr)	Cysteic Acid Content* (μmoles/g wool)
1·6 per cent. in water	24	$\left.\begin{array}{r}819\\879\end{array}\right\}849$
1·6 per cent. in acetic acid	24	672
	24	733
1·6 per cent. in formic acid	16	939
	24	981
5 per cent. in water	41	899
5 per cent. in acetic acid	24 (0°C)	707
	7	795
	24	894
	41	902
5 per cent. in formic acid	7 (0°C)	885
	24 (0°C)	947
	41	1007

* Each value is the result of a single analysis for a separate oxidation. Excess oxidizing reagent was removed by freeze-drying.

For wools analysed by polarographic methods on the intact fibre, Leach (1959) has found that the total sulphur is accounted for by the cysteine, cystine, and methionine (cf. Cuthbertson and Phillips 1945) and therefore no unknown sulphur compound is present. During hydrolysis some cystine and cysteine are destroyed, but, provided the time and temperature of colour development is carefully controlled, the experience in this Laboratory is that the estimates by the Shinohara (1935) and polarographic methods (Stricks, Kolthoff, and Tanaka 1954) on hydrolysates are in good agreement (Maclaren, Leach, and O'Donnell, unpublished data).

Although our results with solutions of peracetic acid in water or acetic acid did not indicate quantitative conversion of cysteine plus cystine residues to cysteic acid residues, quantitative oxidation was observed with formic acid as solvent. A solution of peracetic acid in formic acid will be converted to performic acid when equilibrium is reached, and a considerable amount of the oxidized wool dissolves in the excess formic acid. With 5 per cent. peracetic acid in formic acid at 0°C the rate of formation of performic acid is slower than at room temperature so that complete oxidation of wool has occurred in 24 hr but not in 7 hr (Table 4). This suggests that peracetic acid is a less powerful oxidizing agent than performic acid.

Table 5

CYSTEIC ACID CONTENTS OF HYDROLYSATES OF RESIDUAL OXIDIZED WOOL AFTER PERACETIC ACID OXIDATION AND WASHING WITH WATER

All oxidations carried out at 28°C except where indicated

Strength of Peracetic Acid and Solvent	Time of Oxidation (hr)	Cysteic Acid Content* (μmoles/g oxidized wool)
1·6 per cent. in water	24 (room temp.)	722, 742†
1·6 per cent. in acetic acid	24 (room temp.)	725
5 per cent. in water	8	791
	24	795, 772
	48	762
5 per cent. in acetic acid	8	812
	24	873
	48	828

* Each value is the result of a single analysis for a separate oxidation.

† Further oxidation of this preparation (duplicates 739, 745) with performic acid, at 0°C for 17 hr, increased the cysteic acid content to 838 μmoles/g of peracetic acid-oxidized wool (replicates 860, 820, 843, 827).

If the residual oxidized fibre obtained with 1·6 per cent. aqueous peracetic acid contains partial oxidation products of cystine, they should be converted by performic acid to cysteic acid. This was found to be the case, the yield of cysteic acid being increased from 742 to 838 μmoles/g. It is clear from these results that we cannot substantiate the claim of Alexander, Fox, and Hudson (1951) that the cystine that disappears on oxidation with 1·6 per cent. aqueous peracetic acid can be quantitatively accounted for as cysteic acid. The sulphur distribution in the present experiments can be satisfactorily accounted for as follows: the cysteic acid content for the freeze-dried reaction mixture (Table 4) minus the cysteic acid content of the residual peracetic acid-oxidized fibre (allowing for the increase in weight of 1 g wool to 1·035 g oxidized fibre) is $(849 - 742 \times 1{\cdot}035) = 81$ μmoles and represents the cysteic acid lost in the oxidizing solution. This value for the dissolved cysteic acid plus the cysteic acid content on the same residual fibre after further oxidation with performic acid $(838 \times 1{\cdot}035 \quad 867)$ gives a total of 948

μmoles/g wool. This is in good agreement with the value obtained by direct performic acid oxidation of wool (965 μmoles/g) taking into account the fact that the 81 μmoles lost during isolation of the peracetic acid-oxidized fibre might also be increased by further oxidation with performic acid.

It should be emphasized that our results are based on hydrolysates of oxidized wool. Partial oxidation products of disulphides can undergo disproportionation reactions on hydrolysis (Lavine 1936) and cysteic acid may well be an end-product of some of these reactions. The cysteic acid content of hydrolysates may be higher than that of the intact fibre for partially oxidized wools.

TABLE 6

CYSTINE CONTENT OF HYDROLYSATES OF WOOLS OXIDIZED WITH PERACETIC AND PERFORMIC ACIDS

All oxidations carried out at 0°C except where indicated

Reagent	Time of Oxidation (hr)	Cystine Content of Hydrolysate* (μmoles/g oxidized wool)
1·6 per cent. aqueous peracetic acid	24 (room temp.)	27†
		34†
		32†
Performic acid	5	15
	5	15
	6	20†
	18	4†
	21	0

* Each value is the result for a separate oxidation and hydrolysis. In all analyses at least 250-mg samples of oxidized wool were hydrolysed and analysed by an adaptation of the method of Stricks, Kolthoff, and Tanaka (1954) by Dr. S. J. Leach as described by Human (1958).

† These values were for residual fibre samples from large-scale oxidations.

To account for the analytical values it is obvious that the 1 per cent. of nitrogen lost during isolation of the oxidized fibre (and containing 81 μmoles cysteic acid) using aqueous 1·6 per cent. peracetic acid constitutes a sulphur-rich fraction. This sulphur-rich fraction was not obtained when 1·6 per cent. solutions of peracetic acid in acetic acid were used. For higher concentrations of peracetic acid in acetic acid there was always considerably less material dissolved during isolation of the oxidized fibre (Table 2) than was the case with the corresponding aqueous solutions. These results suggest that with the aqueous solutions containing hydrogen peroxide some random splitting of peptide bonds has occurred to give more low molecular weight material. The increased proportion of dialysable material in γ-keratose prepared using 1·6 per cent. aqueous peracetic acid compared with γ-keratose obtained from performic acid-oxidized wool (O'Donnell and Thompson 1959) supports this conclusion.

(d) *Cystine Contents of Wool Oxidized with Performic and Peracetic Acids*

The oxidized wools studied here contained no cystine in the intact fibre as measured by the method of Leach (1959). However, the presence of partial oxidation products leads to the formation of cystine on hydrolysis (Lavine 1936). Polarographic analyses (Stricks, Kolthoff, and Tanaka 1954) for cystine in hydrolysates of oxidized wools gave the values shown in Table 6. Oxidation is only complete after 21 hr of reaction with performic acid when no cystine could be detected.

V. Discussion

From the data presented in this paper it is apparent that performic acid is a more powerful oxidizing agent than peracetic acid. Oxidized wool was prepared with 1·6 per cent. aqueous peracetic acid under conditions reported by Alexander, Hudson, and Fox (1950) and Corfield, Robson, and Skinner (1958) to lead to complete loss of cystine. We found that this material had a cysteic acid content which was increased by 13 per cent. on further oxidation with performic acid. This difference cannot be attributed to different rates of diffusion of the reagents into the interior of the fibre since polarographic experiments, according to the method of Leach (1959), on the intact fibre have shown that within 1 hr of oxidation with aqueous peracetic acid very little cystine is detectable. In 98–100 per cent. formic acid, which swells wool fibres 50 per cent. more than in aqueous solutions (Speakman 1933), peracetic acid will not completely oxidize the cystine in 7 hr at 0°C, although reaction is complete in 24 hr. With performic acid in formic acid the reaction is virtually complete in 4 hr at 0°C.

Supporting evidence for the presence of partial oxidation products of cystine in peracetic acid-oxidized wool has been presented by Maclaren, Leach, and O'Donnell (unpublished data). They found that, even after short treatments with peracetic acid, almost complete loss of cystine was evident on polarographic examination of the intact fibre. Treatment of the oxidized wool with alkali gave a 200–300 per cent. increase (compared with that of the intact oxidized fibre) in the cystine content, due to hydrolytic oxidation and reduction of the partial oxidation products (Lavine 1936), while hydrolysis with acid gave a 300–400 per cent. increase in apparent cystine content.

The structure of the partial oxidation products is not known but could include some of the following modifications of the –S–S– bond (Lavine, Toennies, and Wagner 1934; Harris and Smith 1937): $-SO-S-$, $-SO_2-S-$, $-SO_2-SO-$, $-SO_2-SO_2-$, $-SOH$, $-SO_2H$. The increase in weight on oxidation suggests that the more highly oxidized modifications, e.g. $-SO_2H$, are present in the oxidized wools studied here. Moreover, the proportions of α-, β-, and γ-keratoses isolated from wool oxidized either with performic acid or with 1·6 per cent. aqueous peracetic acid are almost identical (O'Donnell and Thompson 1959) suggesting the cross-linking sulphur–sulphur bonds are broken in both cases.

Hydrolysis of most oxidized wools reformed some cystine (Table 6) as measured by the polarographic method. This does not agree with the results of Alexander, Hudson, and Fox (1950) and Corfield, Robson, and Skinner (1958) who used the Shinohara (1935) method and found virtually no cystine in the hydrolysates.

For the isolation of proteins from oxidized wool performic acid is a better oxidizing agent than peracetic acid since oxidation of the modified amino acid residues is complete. Our results suggest that there is also less random hydrolysis of peptide bonds. Moreover, it is known from studies of the action of the enzyme leucine aminopeptidase, which is specific for L-amino acid residues, on the separate chains of oxidized insulin and other oxidized proteins (Hill and Smith 1957), that performic acid oxidation does not cause racemization.

VI. ACKNOWLEDGMENTS

The authors wish to thank Mr. W. B. Hall for the statistical analysis and Dr. S. J. Leach for assistance in the polarographic estimations of cysteine and cystine. Dr. K. W. Zimmerman performed the ash and sulphur analyses.

VII. REFERENCES

ALEXANDER, P., FOX, M., and HUDSON, R. F. (1951).—*Biochem. J.* **49**: 129.

ALEXANDER, P., HUDSON, R. F., and FOX, M. (1950).—*Biochem. J.* **46**: 27.

BIDMEAD, D. S., and LEY, F. J. (1958).—*Biochim. Biophys. Acta* **29**: 562.

CORFIELD, M. C., and ROBSON, A. (1955).—*Biochem. J.* **59**: 62.

CORFIELD, M. C., ROBSON, A., and SKINNER, B. (1958).—*Biochem. J.* **68**: 348.

CUTHBERTSON, W. R., and PHILLIPS, H. (1945).—*Biochem. J.* **39**: 7.

HARRIS, M., and SMITH, A. L. (1937).—*Bur. Stand. J. Res., Wash.* **18**: 623.

HILL, R. L., and SMITH, E. L. (1957).—*J. Biol. Chem.* **228**: 577.

HIRS, C. H. W. (1956).—*J. Biol. Chem.* **219**: 611.

HOMMES, F. A., SANTEMA-DRINKWAARD, J., and HUISMAN, T. H. J. (1956).—*Biochim. Biophys. Acta* **20**: 564.

HUMAN, J. P. E. (1958).—*Text. Res. J.* **28**: 647.

JIRGENSONS, B., and IKENAKA, T. (1958).—Abstr. Pap. 134th Meet. Amer. Chem. Soc. Chicago, Sept., 1958. p. 34c.

JOLLÈS-THAUREAUX, J., JOLLÈS, P., and FROMAGEOT, C. (1958).—*Biochim. Biophys. Acta* **27**: 298.

KIMMEL, J. R., THOMPSON, E. O. P., and SMITH, E. L. (1955).—*J. Biol. Chem.* **217**: 151.

LAVINE, T. F. (1936).—*J. Biol. Chem.* **113**: 583.

LAVINE, T. F., TOENNIES, G., and WAGNER, E. C. (1934).—*J. Amer. Chem. Soc.* **56**: 242.

LEACH, S. J. (1959).—*Biochim. Biophys. Acta* **33** (in press).

LI, C. H. (1957).—*J. Biol. Chem.* **229**: 157.

MEEDOM, B. (1956).—*Acta Chem. Scand.* **10**: 881.

MOORE, S., and STEIN, W. H. (1948).—*J. Biol. Chem.* **176**: 367.

MOORE, S., and STEIN, W. H. (1954).—*J. Biol. Chem.* **211**: 893.

O'DONNELL, I. J., and THOMPSON, E. O. P. (1959).—*Aust. J. Biol. Sci.* **12**: 294.

SANGER, F. (1949*a*).—*Biochem. J.* **44**: 126.

SANGER, F. (1949*b*).—*Biochem. J.* **45**: 563.

SCHRAM, E., MOORE, S., and BIGWOOD, E. J. (1954).—*Biochem. J.* **57**: 33.

Schroeder, W. A., Kay, L. M., Lewis, B., and Munger, N. (1955).—*J. Amer. Chem. Soc.* **77**: 3901.

Shinohara, K. (1935).—*J. Biol. Chem.* **112**: 671, 683.

Simmonds, D. H. (1956).—Proc. Int. Wool. Text. Res. Conf. Aust. 1955. Vol. C. p. C-65.

Speakman, J. B. (1933).—*J. Soc. Leath. Tr. Chem.* **17**: 229.

Stricks, W., Kolthoff, I. M., and Tanaka, N. (1954).—*Anal. Chem.* **26**: 299.

Swern, D. (1953).—"Organic Reactions." Vol. 7. p. 378. (J. Wiley & Sons, Inc.: New York.)

Thompson, E. O. P. (1954).—*Biochim. Biophys. Acta* **15**: 440.

Thompson, E. O. P. (1956).—Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. C. p. C-102.

Thompson, E. O. P. (1958).—*Biochim. Biophys. Acta* **29**: 643.

Toennies, G., and Homiller, R. P. (1942).—*J. Amer. Chem. Soc.* **64**: 3054.

Wilcox, P. E., Cohen, E., and Tan, W. (1957).—*J. Biol. Chem.* **228**: 999.

STUDIES ON OXIDIZED WOOL

II. EXTRACTION OF SOLUBLE PROTEINS FROM WOOL OXIDIZED WITH PERFORMIC ACID

By I. J. O'Donnell* and E. O. P. Thompson*

[*Manuscript received April* 9, 1959]

Summary

The variables involved in the alkaline extraction of wool oxidized with performic acid have been studied and reproducible methods of extraction have been developed. The presence of potassium sulphate reduces the amount of protein extracted but, for maximal precipitation of protein at pH 4, potassium sulphate is necessary. The extraction has been followed by estimating the cysteic acid and nitrogen contents of the soluble material. It is shown that the fractions of oxidized wool—α-+γ-, α-, and γ-keratose—contain approximately 9, 5, and 16 g cysteic acid nitrogen per 100 g total nitrogen respectively, whether extraction is carried out at pH 8 or 11.

Comparison is made with wool oxidized with aqueous peracetic acid. The α- and γ-keratoses from the ammonia extraction of peracetic acid-oxidized wool contain less cysteic acid than those from the performic acid-oxidized wool, signifying less complete oxidation of disulphide bonds by peracetic acid. The dialysable nitrogen in the ammonia extract is higher when aqueous peracetic acid replaces performic acid indicating greater splitting of peptide bonds with peracetic acid. No evidence is obtained for the preferential extraction of a sulphur-rich fraction following performic acid oxidation of wool.

I. Introduction

The heterogeneity of wool fibres as revealed by histological techniques makes it obvious that some fractionation procedure is necessary before chemical characterization of the various components can be achieved. Some progress has been made in separating special histological components, such as the scale cells (Bradbury 1959), but for a knowledge of the major constituent proteins it is necessary to extract them from the fibre and attempt purification by the methods of protein chemistry. To dissolve much of the wool protein material, without hydrolysis of peptide bonds, it is first necessary to break the disulphide linkages of cystine residues.

By fractional extraction with thiols in alkaline solutions evidence for the presence of several components has been obtained (Gillespie and Lennox 1955) and one of these components has been extensively studied (Gillespie 1957). Reduction techniques such as this have an advantage in that they usually modify only the cystine residues. A disadvantage, however, is the fact that the reaction is reversible and special conditions are necessary to ensure complete reduction. Moreover, stabilization of the thiol groups with alkylating agents, such as iodoacetic acid,

---

*Division of Protein Chemistry (formerly Biochemistry Unit), C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

is essential if oxidation and polymerization reactions are to be avoided. This alkylation reaction may cause side reactions if not carefully controlled (Moore *et al.* 1959).

Examination of tryptic digests of a fractionated sample of *S*-carboxymethylkerateine 2 (Gillespie 1957) by the methods of Hirs, Moore, and Stein (1956) revealed a complex mixture of ninhydrin-positive peaks which did not analyse for amino acids in stoichiometric proportions (Thompson, unpublished data). To explain the complexity of these results it is necessary to assume either that the extracted protein was a complex mixture of closely related proteins with similar physical properties, or that racemization of some amino acid residues had occurred under the conditions, pH 10·5–12·3 at 50°C, used to selectively extract the protein. A study of the proteins isolated from oxidized wool has been undertaken to gather evidence on these two alternative explanations.

The extraction of soluble proteins from wool oxidized with peracetic acid has been studied by Alexander and co-workers (Alexander and Hudson 1954; Alexander and Smith 1956) who treated the oxidized wool with ammonia solution. Some 10 per cent. (β-) of the wool remained undissolved and the soluble protein was fractionated into acid-precipitable (α-, 60 per cent.) and non-acid-precipitable (γ-, 30 per cent.) components.* The amino acid compositions of these fractions have been determined (Corfield, Robson, and Skinner 1958) as well as their *N*-terminal amino groups (Alexander and Smith 1956).

In Part I of the series (Thompson and O'Donnell 1959) it has been shown that performic acid completely oxidizes the cystine residues of wool to cysteic acid residues whereas dilute aqueous peracetic acid does not. With this reagent there is no evidence of racemization (Hill and Smith 1957). Furthermore, the action of performic acid on proteins has been extensively investigated (e.g. Sanger 1949; Hirs 1956) and its action is not complicated by the presence of much free peroxide. In the case of peracetic acid, hydrogen peroxide is formed on dilution with water and it is known that hydrogen peroxide can break peptide bonds (O'Donnell and Woods 1956). For these reasons we have chosen for study wool oxidized with performic acid in formic acid (performic acid reagent). Oxidized wool prepared in this way is not as easy to handle as the peracetic acid product due to its partial solution in the performic acid reagent, but techniques for handling large quantities of wool have been developed.

This paper describes the extraction of soluble proteins from wool oxidized with performic acid and the variables involved. Comparison was made with proteins extracted from wool oxidized with dilute aqueous peracetic acid. The extraction and fractionation procedure was followed by analyses for nitrogen and cysteic acid contents.

II. Materials and Methods

The wool and oxidizing agents used were those as described in Part I (Thompson and O'Donnell 1959).

---

* The terms α-, β-, and γ-keratoses were introduced by Alexander (Alexander and Hudson 1954). In this paper, while retaining this nomenclature, we consider they are operational definitions rather than definite compounds. "Acid-precipitable" refers to material precipitated at pH 4 in the presence of 0·1M potassium sulphate.

Nitrogen contents of solutions of the proteins and peptides were determined by the Kjeldahl nitrogen procedure (Chibnall, Rees, and Williams 1943). After overnight digestion the nitrogen content of dry wool was 16·9 g/100 g.

When ammonia was present in the protein solution this was first removed by evaporation overnight in the presence of potassium carbonate in a vacuum over concentrated sulphuric acid (Moore and Stein 1951).

For cysteic acid analyses the proteins were hydrolysed overnight under reflux with 6N hydrochloric acid and analysed as described by Thompson and O'Donnell (1959).

III. EXPERIMENTAL

(a) *Oxidation of Wool with Performic and Peracetic Acids*

Ten 1-g samples of wool previously cooled to 0°C were each left at 0°C with 30 ml of performic acid reagent for 6 and 16 hr. The wool swelled and partially dissolved. After the completion of oxidation it was poured into 50 ml of distilled water in a dialysis bag and dialysed for 48 hr against running tap water to remove the formic acid and peroxide. This dialysed mixture was then stored in the frozen state until required.

Peracetic acid oxidation of wool was carried out as described by Alexander and Smith (1956). The wool was stood (liquor–wool ratio 50 : 1) with 1·6 per cent. (w/v) aqueous peracetic acid (diluted from the Laporte 40 per cent. (w/v) product) for 24 hr at room temperature with occasional shaking. After this it was thoroughly washed and air-dried.

The percentage of dialysable γ-keratose was always measured after dialysis in 20/32 Visking "Cellophane" tubing (Kjeldahl nitrogen determinations).

(b) *Extraction of Soluble Proteins from Oxidized Wool*

Oxidized wools (1-g samples) were stirred with approximately 150 ml water at various pH values, the pH being maintained by using either ammonia or potassium hydroxide by means of a pH-stat. Subsequently, before standing for extraction, the volume was increased to 200 ml. The amount of material extracted under various conditions was studied; these conditions included time of standing, pH, treatment with Waring Blendor or glass beads, successive extractions, time of standing before homogenizing, presence and absence of potassium sulphate during extraction, and nature of alkali used.

The supernatant (α- + γ-keratoses) was separated from the undissolved material by centrifugation at 24,000 *g* and subsequent filtration. The extracted (α- + γ-) keratoses were then made 0·1M with respect to potassium sulphate, and glacial acetic acid added to bring the pH to 4; the insoluble α-keratose was separated by centrifugation and was dissolved again at its pH of extraction. It was concentrated, after dialysis to free it from potassium sulphate by pervaporation and stored as a frozen 1 per cent. aqueous solution.

When it was desired to prepare γ-keratose free from salt the oxidized wool was extracted with ammonia, and ammonium acetate was used in place of potassium sulphate before addition of acetic acid. α-, β-, and γ-keratoses were then obtained in the dried, salt-free form by freeze-drying followed by standing in a vacuum over concentrated sulphuric acid and sodium hydroxide pellets to remove the ammonium acetate and acetic acid.

When 0·3M ammonium acetate or potassium chloride was used in place of 0·1M potassium sulphate the supernatant containing γ-keratose was more cloudy than when potassium sulphate was used and needed longer high-speed centrifugation for its clarification.

TABLE 1

EXTRACTION OF OXIDIZED WOOL AT VARIOUS pH VALUES

Values are per cent. nitrogen

Nitrogen Expressed as:	Wool Oxidized with Performic Acid at pH:					Wool Oxidized with Peracetic Acid at pH:
	6·5	8	9	10	11	11
Extracted nitrogen as per cent. initial wool nitrogen (α- + γ-keratose)	34	48, 53, 53, 49, 52, 47, 58, 51	54	60	79, 79, 82, 82, 81, 82, 79, 75, 76, 78	74
Non-acid-precipitable nitrogen (γ-keratose) as per cent. nitrogen extracted	48	36	38	39	35	33
Dialysable nitrogen as per cent. nitrogen extracted	—	6	5	8	6	12
Dialysable nitrogen as per cent. non-acid-precipitable nitrogen	—	17	14	19	17 (indirect) 11 (direct)	36 (indirect) 22 (direct)

IV. RESULTS

(a) *Variables in the Extraction Process*

(i) *Effect of Alkali Used.*—It was found that ammonia gave no better extraction at pH 11 than did potassium hydroxide in the absence of potassium sulphate, the amount extracted in 24 hr at 2°C being 75–82 per cent. (Table 1) of the total nitrogen. Our work was mainly carried out using potassium hydroxide, since this was more convenient for Kjeldahl nitrogen determinations; however, in the absence of a pH-stat ammonia solution has the advantage that it buffers at pH 11 and is consequently more convenient.

(ii) *Effect of Potassium Sulphate.*—The presence of potassium sulphate reduced the rate of extraction of oxidized wool with alkali and also lowered the yield of soluble material (Table 2).

The effect of the presence of this salt during extraction is certainly dramatic. This retardation in rate of extraction and lowering of its final value appears to be connected with the reduced swelling of the oxidized fibres at pH 11 in the presence of the salt in contrast with the marked swelling in its absence. This reduced swelling may result from the protective effect of the salt on the repulsion between the charged acidic groups in the oxidized wool. In the absence of the salt the oxidized fibre swells (and readily fractures in a blendor) and empties its contents into solution (Plate 1, Fig. 1). In the presence of the salt the less swollen fibre is not so readily dispersed (Plate 1, Fig. 2).

TABLE 2

EFFECT OF POTASSIUM SULPHATE ON THE EXTRACTION OF PERFORMIC ACID-OXIDIZED WOOL AT pH 11

pH adjusted with potassium hydroxide

Extract No.	Time of Extraction (hr)	Values are for Nitrogen Expressed as Percentage Total Initial Wool Nitrogen			
		Without Blendor		With Blendor	
		No K_2SO_4	$0 \cdot 1$M K_2SO_4	No K_2SO_4	$0 \cdot 1$M K_2SO_4
1	2	61	37	79 (16 hr)	60
2	16	9	8		
3, 4, 5	48	5	5		
Total extracted:		75	50	79	60

If protein is extracted in the absence of the salt and the salt added before separation of the undissolved β-keratose by centrifugation the percentage of soluble protein extracted is only some 2–3 per cent. lower than when the salt is entirely absent.

When oxidized wool, extracted in the absence of the salt, is centrifuged in a "Servall" centrifuge at approximately 24,000 g the undissolved material does not pack down well, but if $0 \cdot 1$M potassium sulphate is added before centrifugation it packs down very tightly. Here again the shielding of repulsive charges by the salt is possibly the explanation.

(iii) *Effect of Homogenizing in a Waring Blendor.*—Treatment of the swollen fibres for 2 min in a Waring Blendor increased the rate of extraction of the protein, but here also the presence of potassium sulphate caused the extraction to be slower and the ultimate limit lower in the presence of potassium sulphate than in its absence (Table 2). Despite homogenization the extraction was still slow, 69 per cent. of the nitrogen being extracted in 2 hr and 80 per cent. in 24 hr. The ultimate extractability of the wool was not affected by homogenizing.

With one extraction for 16–24 hr at pH 11, with or without homogenizing, some 75–82 per cent. of the nitrogen was extracted. With successive extractions this could be raised to 86 per cent. but beyond this level a higher pH or prolonged extraction is required.

(iv) *Effect of Shaking with Glass Beads.*—Because homogenizing was effective in breaking up the swollen fibres (more than one homogenizing had no enhanced effect) it was thought that if the fibres could be broken up more finely there might be a higher percentage extraction. Shaking 40 ml of protein suspension at pH 11 for 6 hr with 20 g of either 4-mm, 1-mm, or smaller beads showed that the 4-mm and 1-mm beads broke up the fibres much more effectively than the smaller ones. Nevertheless, glass beads did not improve the extractability in 24 hr over that obtained by either standing alone or by homogenizing. Since solutions being extracted are stood overnight in any case, the use of a Waring Blendor is probably superfluous as far as extractability is concerned. However, it has the advantage of making the residual keratin easier to separate by centrifugation from the solution of $\alpha+\gamma$-keratose.

(v) *Effect of Time of Swelling.*—The time of swelling before homogenizing can cause some variation in the final amount of nitrogen extracted. If the time of swelling at pH 8 (followed by 2 min treatment in the Waring Blendor and 24 hr standing at 2°C) was $1\frac{3}{4}$, 4, and 17 hr then the percentage of nitrogen extracted was 48, 51, and 52 per cent. respectively, while the corresponding figures at pH 11 for 25, 33, and 63 min swelling were 76, 75, and 78 per cent. respectively.

(*b*) *Comparisons of Performic and Peracetic Acid-oxidized Wools*

With the performic acid-oxidized wool it was found that 0·8 per cent. of the total nitrogen was lost on dialysis during its preparation, while for 1·6 per cent. aqueous peracetic acid-oxidized wool 1 per cent. of the nitrogen was lost in the supernatant and washings, this latter value agreeing with that obtained by Corfield, Robson, and Skinner (1958). In the performic acid-oxidation of wool some 40 per cent. of the nitrogen passes into solution in the formic acid. However, after dilution with water followed by dialysis (final pH approx. 4·5) only 4·5 per cent. of the nitrogen remained in the supernatant.

In a single extraction of peracetic acid-oxidized wool, 74 per cent. of the total nitrogen was extracted in 16 hr at pH 11 (KOH) with 2 min homogenizing; this value is very close to that of 75–82 per cent. obtained for the performic acid product. These values can both be increased somewhat by successive extractions.

(*c*) *Separation of Extracted Wool Protein into α- and γ-Fractions*

When potassium hydroxide was used for the extraction of oxidized wool and acetic acid was added to bring the pH to 4 it was found there was only a small, variable amount of precipitate. The supernatant was opalescent and could not be clarified by low-speed centrifugation. The addition of 0·1M potassium sulphate before the acetic acid caused the percentage of α-keratose precipitated to increase and to be reproducible, and the supernatant could then be readily clarified by centrifugation. Hence all extracts were made 0·1M in potassium sulphate before acidification.

This problem did not arise with ammoniacal extracts of Alexander and Smith (1956) and Corfield, Robson, and Skinner (1958) since they used 0·1N or 3N ammonia solution which would have given rise to adequate salt for complete precipitation of α-keratose on taking the pH to 4 with acetic acid.

(d) *Extraction of Oxidized Wools at Various pH Values and their Fractionation into α- and γ-Keratoses*

These extractions were routinely carried out by standing the oxidized wool in potassium hydroxide for 1–4 hr with the required pH being maintained with a pH-stat, the time of standing varying with the pH. At pH 11 the wool was swollen and wet in a few minutes but at pH 6–8, 3–4 hr were required. The suspension was then homogenized for 2 min and allowed to stand 16–24 hr at 2°C before centrifugation.

The combined α-+γ-keratoses were made 0·1M with respect to potassium sulphate and brought to pH 4 with a few drops of glacial acetic acid. The precipitate was centrifuged off and the pellet of α-keratose redissolved, with stirring, at the pH at which it had been extracted from the fibre. This redissolution may take several hours. The solution of γ-keratose was concentrated by freeze-drying.

The values for percentage extraction at various pH values are given in Table 1. With increasing pH the percentage of nitrogen extractable increases. At pH 8 the amount extracted was 52 per cent. and this did not increase above 58 per cent. on two further extractions of the remaining material. Moreover, taking the pellet of remaining protein to pH 11 at this stage only brought the total extraction to 69 per cent. as compared with the 75–82 per cent. normally obtained if the wool was extracted only at pH 11. The wool extracted at pH 8 does not have the exploded appearance of Plate 1, Figure 1, and, even on then taking to pH 11, it does not attain this state. It seems that if oxidized wool is extracted at pH 11 the large immediate osmotic effect causes the fibres to swell rapidly and release their protein. At pH 8 the effect is much smaller and by the time 50 per cent. of the protein has dissolved at this lower pH the cause of the large osmotic effect at pH 11 is lost.

The percentage of extracted material which is γ-keratose is sensibly constant in the range pH 8–11 and slightly higher at pH 6·5. About 16 per cent. of the γ-keratose is dialysable at all the pH values studied. This value is much lower than the value of 36 per cent. obtained by us and the 82 per cent. (Alexander and Smith 1956) obtained for γ-keratose prepared from peracetic acid-oxidized wool. The indirect values for dialysable γ-keratose in the table were obtained from the difference in nitrogen contents on undialysed and dialysed (α-+γ-) solutions as per cent. non-acid-precipitable nitrogen. We cannot account for the difference between the direct and indirect values.

Reprecipitation (in the presence of potassium sulphate) of α-keratose after solution at pH 11 showed that only 2 per cent. of this redissolved material was non-acid-precipitable.

(e) *Cysteic Acid Contents of α- and γ-Keratoses*

Table 3 shows the cysteic acid contents of the hydrolysates of α- and γ-keratoses extracted at various pH values. The values for α- and γ-keratose are higher than those obtained by Corfield, Robson, and Skinner (1958) and by us using aqueous peracetic acid. This is not surprising in view of the incomplete oxidation with peracetic acid (Thompson and O'Donnell 1959).

There is very little difference between the values for α-keratose at pH 6·5, 8, and 11 and only small differences in the γ-keratose values.

Table 3

CYSTEIC ACID CONTENTS OF α- AND γ-KERATOSES

Values* are for nitrogen expressed as percentage total nitrogen

Oxidation with:	pH of Extraction	α- + γ-Keratoses	α-Keratose	γ-Keratose
Performic acid	6·5	10·2	5·6	16·5
	8	8·9	5·3	15·3 15·4 } 15·3
	11	9·0	5·4 5·2 } 5·2 5·0	16·1
Peracetic acid	11†		3·72	14·5
	11‡		4·7 4·7 } 4·7	

*Each value is the mean of duplicate estimations for a separate preparation. The original wool had an equivalent cysteic acid content of 979 μ moles/g. This is 8·1 g nitrogen per 100 g of total nitrogen.

†Corfield, Robson, and Skinner (1958).

‡Two batches of α-keratose extracted at pH 11 were kindly supplied by Mr. E. F. Woods. Higher values were obtained with these samples before reprecipitation at pH 4, probably due to contamination with γ-keratose.

V. Discussion

The cysteic acid sulphur of oxidized wool accounts for 95 per cent. of the total sulphur (Thompson and O'Donnell 1959) and analyses for cysteic acid afford a convenient method for following the sulphur distribution in the isolated fractions.

It appears that, if cysteic acid content (Table 3) and non-acid-precipitability (Table 1) be taken as characteristics for γ-keratose, the proportion of γ-keratose extracted (as a percentage of the total amount extracted) is the same in the pH range 8 to 11 and only slightly higher at pH 6·5. Certainly there is no indication of a large preferential extraction or a considerable amount of "sulphur-rich" (i.e. γ-) fraction emerging first. It is difficult to reconcile these results with those of Corfield, Robson, and Skinner (1958), who, by gradient extraction of peracetic acid-oxidized wool with 0·2M phosphate in the pH ranges 4·5–7 and 9–11·5, obtained two extracts differing in solubility, and in the approximate quantitative amounts expected for γ- and α-keratoses.

We have explored the extraction of oxidized wool at pH 8 because of a preference for working at pH values near neutrality where dangers of racemization and peptide bond hydrolysis of extracted protein are minimized. At pH's above 10 the extracted protein solution takes on a yellow colour which is possibly due to the action of alkali on the oxidized tryptophan residues. The work reported here indicates that the 50 per cent. of protein extracted at pH 8 is not different from the 80 per cent. extracted at pH 11 (Tables 1 and 3).

The percentage of γ-keratose which is dialysable is much less for γ-keratose prepared from performic acid-oxidized wool than for material prepared from aqueous peracetic-acid oxidized wool. This may point to more severe degradation of wool in the form of peptide-bond breakage when using aqueous peracetic acid (cf. Thompson and O'Donnell 1959). On the other hand, if this were the case it might be expected that the proportion of γ-keratose in the extracts from peracetic acid-oxidized wool would be higher than for performic acid-oxidized wool. The equality of the proportion of γ-keratoses in both extracts in view of the more severe action of peracetic acid may arise from incomplete oxidation of the cystine sulphur–sulphur linkages in the wool by peracetic acid (Thompson and O'Donnell 1959; Maclaren, Leach and O'Donnell, unpublished data).

VI. ACKNOWLEDGMENT

The authors wish to thank Mr. L. West for Kjeldahl nitrogen determinations.

VII. REFERENCES

ALEXANDER, P., and HUDSON, R. F. (1954).—"Wool: Its Chemistry and Physics." (Chapman and Hall Ltd.: London.)

ALEXANDER, P., and SMITH, L. F. (1956).—Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. B. p. B-56.

BRADBURY, J. H. (1959).—*Nature* **183**: 305.

CHIBNALL, A. C., REES, M. W., and WILLIAMS, E. F. (1943).—*Biochem. J.* **37**: 354.

CORFIELD, M. C., ROBSON, A., and SKINNER, B. (1958).—*Biochem. J.* **68**: 348.

GILLESPIE, J. M. (1957).—*Aust. J. Biol. Sci.* **10**: 105.

GILLESPIE, J. M., and LENNOX, F. G. (1955).—*Aust. J. Biol. Sci.* **8**: 97.

HILL, R. L., and SMITH, E. L. (1957).—*J. Biol. Chem.* **228**: 577.

HIRS, C. H. W. (1956).—*J. Biol. Chem.* **219**: 611.

HIRS, C. H. W., MOORE, S., and STEIN, W. H. (1956).—*J. Biol. Chem.* **219**: 623.

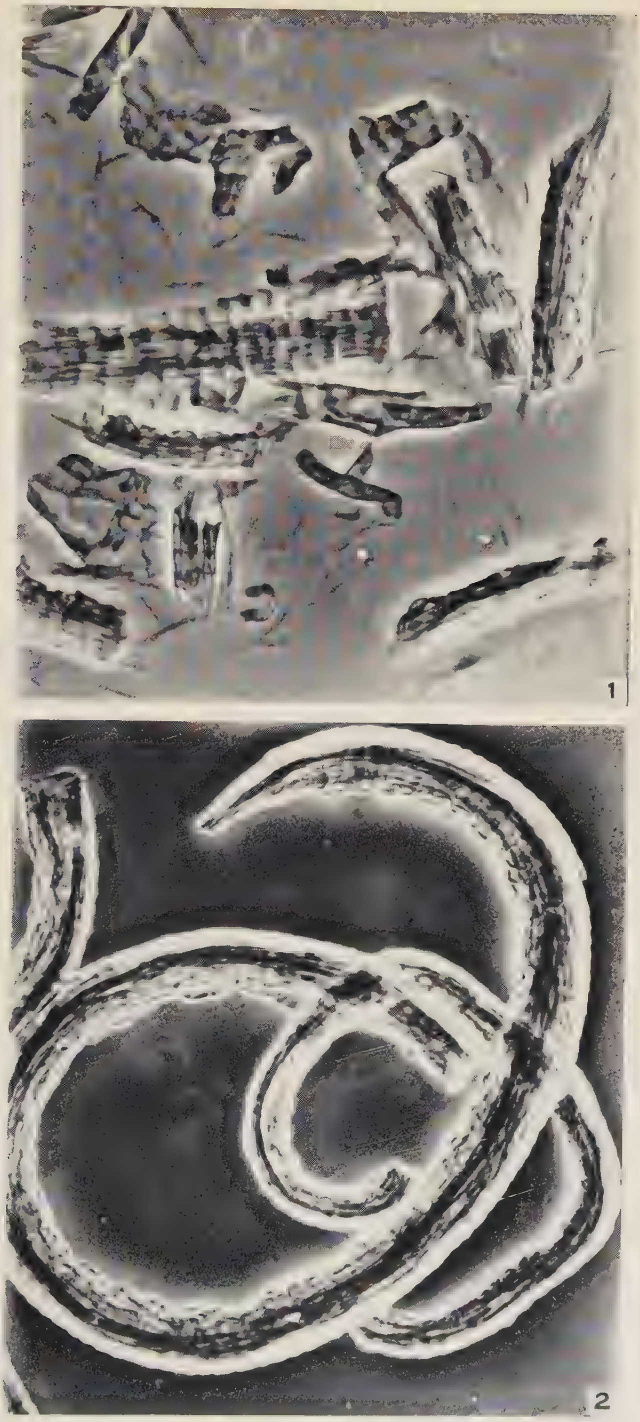
MOORE, S., COLE, R. D., GUNDLACH, H. G., and STEIN, W. H. (1959).—"Symposium on Proteins." 4th Int. Congr. Biochem., Vienna, 1958. (In press.)

MOORE, S., and STEIN, W. H. (1951).—*J. Biol. Chem.* **192**: 663.

O'DONNELL, I. J., and WOODS, E. F. (1956).—Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. B. p. B-48. See also discussion, p. B-273.

SANGER, F. (1949).—*Biochem. J.* **44**: 126.

THOMPSON, E. O. P., and O'DONNELL, I. J. (1959).—*Aust. J. Biol. Sci.* **12**: 282.



Fibres of performic acid-oxidized wool after extraction at pH 11 and homogenization in a Waring Blendor in the absence of potassium sulphate (Fig. 1) and in the presence of 0·1M potassium sulphate (Fig. 2).

A METHOD FOR THE ISOLATION OF DNP-CYSTEIC ACID AND ITS APPLICATION TO THE DETERMINATION OF *N*-TERMINAL HALF-CYSTINE RESIDUES IN WOOL

By E. O. P. Thompson*

[*Manuscript received March* 18, 1959]

Summary

A paper ionophoretic method is described for the isolation of dinitrophenyl (DNP)-cysteic acid from hydrolysates of oxidized DNP-proteins.

DNP-wool after oxidation and hydrolysis is shown to contain DNP-cysteic acid indicating the presence of *N*-terminal half-cystine or cystine residues. These residues, 6·7 μmoles/g, comprise the second most abundant terminal group in wool.

I. Introduction

The 1,2,4-fluorodinitrobenzene (Sanger 1945) and phenyl*iso*thiocyanate (Edman 1950) methods of *N*-terminal group analysis for proteins cannot successfully be applied to the detection of *N*-terminal cysteine, half-cystine, or cystine residues since destruction of the dinitrophenyl (DNP) (Porter and Sanger 1948) and phenylthiohydantoin (PTH) (Edman 1950) derivatives occurs during hydrolysis of the DNP- or phenylthiocarbamyl protein. However, if the cysteine, cystine, or half-cystine residues are first converted by oxidation to β-sulphoalanyl (cysteic acid) residues (Bettelheim 1955; Edman and Lauber 1956), or by reduction and alkylation to *S*-carboxymethylcysteine residues (Edman and Lauber 1956) the corresponding DNP- or PTH-derivatives are stable.

A method for the determination of *N*-terminal half-cystine residues in chymotrypsinogen was described by Bettelheim (1955) in which the DNP-chymotrypsinogen was oxidized with performic acid and the DNP-cysteic acid liberated on hydrolysis was separated from the other DNP-compounds and estimated. Ether extraction removed dinitrophenol and DNP-amino acids other than DNP-cysteic acid and ε-DNP-lysine which remained in the aqueous phase together with the free amino acids.

Separation of the small amount of DNP-cysteic acid from these compounds is usually necessary before satisfactory paper chromatograms can be obtained for the isolation of the DNP-cysteic acid. For this purpose Bettelheim (1955) used a sulphonated polystyrene ion-exchange resin which formed insoluble salts with the amino acids and ε-DNP-lysine but which does not bind DNP-cysteic acid by salt linkage due to its net negative charge conferred by the low pK value for the sulphonic acid group. However, adsorption due to secondary valence forces can occur with DNP-compounds (Anfinsen, Sela, and Tritch 1956) and could lead to low recoveries of DNP-cysteic acid.

---

* Division of Protein Chemistry (formerly Biochemistry Unit), C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

Preliminary experiments on hydrolysates of DNP-wool which had previously been oxidized with performic acid showed that DNP-cysteic acid was present but difficulties were experienced in obtaining satisfactory paper chromatograms due to streaking and to the presence of other yellow zones on the paper. For quantitative estimates an alternative method of separating DNP-cysteic acid from the hydrolysate of oxidized DNP-wool was developed which utilized selective migration of the DNP-cysteic acid during paper ionophoresis. In 1N formic acid, pH 1·8, sulphonic groups are ionized whereas carboxyl groups are unionized. The negatively charged DNP-cysteic acid migrates to the anode during ionophoresis while ε-DNP-lysine and amino acids, being positively charged, migrate in the opposite direction. In developing this method, which proved preferable to paper or column chromatography, experiments were conducted on DNP-chymotrypsinogen, which bears an *N*-terminal half-cystine residue (Bettelheim 1955), and on DNP-plasma albumin. Plasma albumin was reported to contain *N*-terminal half-cystine (Titani, Yoshikawa, and Satake 1956) but this could not be confirmed using the new method, whereas under similar conditions the *N*-terminal half-cystine residue in chymotrypsinogen was readily detected (Thompson 1958).

Peracetic acid was recommended by Alexander, Hudson, and Fox (1950) for the oxidation of wool, since it is more stable in aqueous solution than performic acid, has a similar specificity, and does not dissolve much of the fibre. It has not been used in these studies since comparative experiments on wool oxidized with peracetic and performic acids have shown that oxidation with peracetic acid is incomplete. Much lower yields of cysteic acid (Thompson and O'Donnell 1959) were obtained in hydrolysates of wool oxidized with peracetic acid than was the case with performic acid.

N-terminal cystine or half-cystine residues have not previously been detected in wool but quantitative estimations described in this paper now show these residues to be the second most abundant *N*-terminal group.

II. MATERIALS AND METHODS

DNP-wool was prepared from virgin Merino wool, 64's quality, by the method of Fritze and Zahn (1956) using the wools previously described (Thompson 1957).

III. EXPERIMENTAL

(*a*) *Oxidation of DNP-wool with Performic Acid*

Performic acid reagent was prepared by mixing 1 vol. 30 per cent. hydrogen peroxide and 9 vol. 98–100 per cent. formic acid and allowing to stand 1–2 hr at room temperature.

After cooling to 0°C, sufficient performic acid reagent to oxidize at least 10 times the content of cystine, cysteine, methionine, and tryptophan in wool (theoretical quantity 3 ml per g wool) was added to the DNP-wool previously cooled to 0°C. The reaction mixture was kept at 0°C for 16–24 hr to ensure maximum oxidation.

Most of the excess reagent was removed in a rotary evaporator at temperatures below 30°C, then 10 vol. of ice-cold water was added and the mixture freeze-dried.

The residue was mixed with a smaller quantity of cold water and the freeze-drying repeated to ensure complete removal of hydrogen peroxide.

Only a relatively small amount of DNP-wool was soluble in the performic acid reagent in contrast to wool, 40 per cent. of which may dissolve during oxidation at 0°C for 24 hr.

(*b*) *Hydrolysis of DNP-wool and Extraction of DNP-cysteic Acid*

The oxidized DNP-wool (400 mg) was hydrolysed with 6N HCl (15 ml) in an oil-bath at 138–140°C for 8 hr. The hydrolysate was diluted with water to 25 ml and extracted with peroxide-free ether (3×25 ml). The combined extracts were washed with water (3×5 ml). The combined aqueous phase and washings was extracted with 10-ml portions of *n*-butanol. Three extractions were usually sufficient to remove the yellow colour. The combined butanol extracts were washed with water (2×5 ml) and the butanol removed in a rotary evaporator *in vacuo* at as low a temperature as possible. The residue was dissolved in aqueous acetone and diluted to 5 ml.

(*c*) *Paper Ionophoretic Separation of DNP-cysteic Acid*

Suitable samples (usually 1 ml) of the aqueous acetone solution containing DNP-cysteic acid were concentrated by evaporation in an air stream and applied as a band 2 in. long to the middle of a sheet of Whatman (No. 3 MM) filter paper 58 cm long, by means of a capillary tube. The sheet was folded and placed in a hanging-strip ionophoresis apparatus similar to that described by Durrum (1951). Platinum wire electrodes were used with 1N formic acid (pH 1·8) as electrolyte.

The paper was wet with electrolyte to within 1 cm of the zones at the starting line. The zones were concentrated into a narrow band by the liquid front as it rose by capillarity.

A potential difference of 240 V was applied for 16 hr. Only two yellow bands migrated toward the anode. DNP-cysteic acid moved a distance of 11 cm and was accompanied by a yellow artifact zone which migrated further to a distance of 14 cm. A similar yellow artifact was present in hydrolysates of both unoxidized DNP-wool and oxidized DNP-bovine plasma albumin which contain no DNP-cysteic acid as well as in hydrolysates of oxidized DNP-wool. Paper chromatograms developed with *n*-butanol–acetic acid–water (4 : 1 : 1 by volume, aged) and 75 per cent. aqueous phenol–NH_3–HCN showed that the artifact band travelled considerably faster than DNP-cysteic acid (R_F's 0·29 and 0·18 respectively in the first solvent, 0·58 and 0·38 respectively in the second solvent). Paper chromatography of the mixture of compounds extracted into *n*-butanol did not give clearly defined spots even after treatment with "Dowex-50" sulphonated polystyrene resin as in the method of Bettelheim (1955).

(*d*) *Spectrophotometric Estimation of DNP-cysteic Acid*

When the ionophoretic separation was complete the paper was dried at 40°C for several hours. The yellow bands were then cut out and eluted by immersion in 5 ml or more of 1 per cent. $NaHCO_3$ solution for 30 min. The absorption spectrum of each eluate was measured over the range 300–390 mμ in 1-cm cells using a Beckmann DU spectrophotometer. The maximum absorption of DNP-cysteic acid was at 357·5 mμ and the ratio of absorptions at 390 and 360 mμ was 0·61. The corresponding ratio for the artifact was 0·75-0·80. The absorption of eluates of blank pieces of paper were very low in comparison with those of DNP-cysteic acid.

The concentration of DNP-cysteic acid was estimated from the optical density at 360 mμ using a molar extinction coefficient of 17×10^3 litres per mole cm.

(e) *Recovery of DNP-cysteic Acid from bis-DNP-cystine after Oxidation, Hydrolysis, and Ionophoretic Separation*

The quantitative application of the DNP-method requires correction of the experimental values for losses involved in the various operations. The biggest correction is for destruction during hydrolysis of the DNP-protein and an adequate control experiment is difficult to define. In general, the losses are greatest when a DNP-amino acid is hydrolysed in the presence of protein, least when hydrolysed with DNP-protein, and intermediate when hydrolysed alone (Thompson 1954).

An estimate was made of the loss of bis-DNP-cystine during oxidation. Approximately 1 μmole was oxidized with performic acid at 0°C and the DNP-cysteic acid was separated by ionophoresis and estimated as described. A control sample was purified by paper chromatography in phosphate buffer pH 6·0 (Levy 1954) and estimated from its absorption in 1 per cent. $NaHCO_3$.

Losses of DNP-cysteic acid under the hydrolysis conditions were measured by hydrolysing equal amounts (approx. 2 μmoles) either alone, in the presence of DNP-wool (300 mg), or with a sample of oxidized DNP-wool (250 mg). In each experiment the DNP-cysteic acid was estimated after isolation by butanol extraction followed by ionophoretic separation.

Removal of hydrogen peroxide from the oxidized DNP-wool is essential because of its destructive effects on DNP-compounds (Bettelheim 1955). In addition to repeated freeze-drying, the DNP-wool was extracted with acetone for 6 hr in a Soxhlet apparatus before adding the sample of DNP-cysteic acid.

IV. RESULTS

The results of analyses for DNP-cysteic acid in hydrolysates of oxidized DNP-wool are shown in Table 1. The first determination was made on DNP-wool prepared from 64's top and the others on the virgin Merino wool, 64's quality, previously examined (Thompson 1957) for *N*-terminal residues. Sample 1 was from DNP-wool previously used while sample 2 was a freshly prepared sample.

Table 2 shows the recovery of DNP-cysteic acid from DNP-cystine. Recoveries of DNP-cysteic acid subjected to various control experiments are also shown. The recovery of DNP-cysteic acid hydrolysed in the presence of oxidized DNP-wool was considerably lower than that in the presence of DNP-wool. The former value has been used to correct the experimental values in Table 1 because it more closely resembles the experimental conditions. The corrected value of 6·7 μmoles per g shown in Table 1 equals the experimental value × the oxidation correction factor × the hydrolysis correction factor, $2 \cdot 09 \times (100/91) \times (100/34 \cdot 5)$. Special efforts were made to completely remove any traces of hydrogen peroxide from the oxidized DNP-wool before the hydrolysis in the presence of added DNP-cysteic acid to ensure that the lower recovery in the presence of oxidized DNP-wool was not caused by this compound. Bettelheim (1955) reported increased destruction of DNP-compounds in the presence of hydrogen peroxide and the recoveries found here are considerably lower than those reported by Bettelheim.

TABLE 1

N-TERMINAL HALF-CYSTINE OR CYSTINE RESIDUES IN WOOL

DNP-wool Sample	Uncorrected Values (μmoles/g wool)		Corrected Mean Value (μmoles/g wool)
64's top	2·10, 2·09		
Virgin 64's			
Sample 1	2·10, 2·27	2·09±0·3*	6·7±1·0*
Sample 2	1·80, 2·18		

* 95 per cent. confidence limits.

TABLE 2

RECOVERY OF DNP-CYSTEIC ACID FROM DNP-CYSTINE DURING VARIOUS OPERATIONS

Operation	Recovery (%)	
Oxidation of bis-DNP-cystine	91	
Hydrolysis, 8 hr, 6N HCl	54	51
	48	
	51	
Hydrolysis in presence of DNP-wool, 8 hr, 6N HCl	60	62
	64	
Hydrolysis in presence of oxidized DNP-wool, 8 hr, 6N HCl	39	34·5
	30	

Figure 1 shows the absorption curves for DNP-cysteic acid and for the yellow artifact which also migrates toward the anode. This artifact, which does not have the absorption spectrum typical of DNP-compounds (Sanger 1949), has been found in hydrolysates of unoxidized DNP-wool and of oxidized DNP-bovine plasma albumin where no DNP-cysteic acid could be detected. Although negatively charged at pH 1·8 this artifact is readily separated from DNP-cysteic acid by ionophoresis and easily distinguished from DNP-cysteic acid from the ratio of the absorption at 390 and 360 mμ.

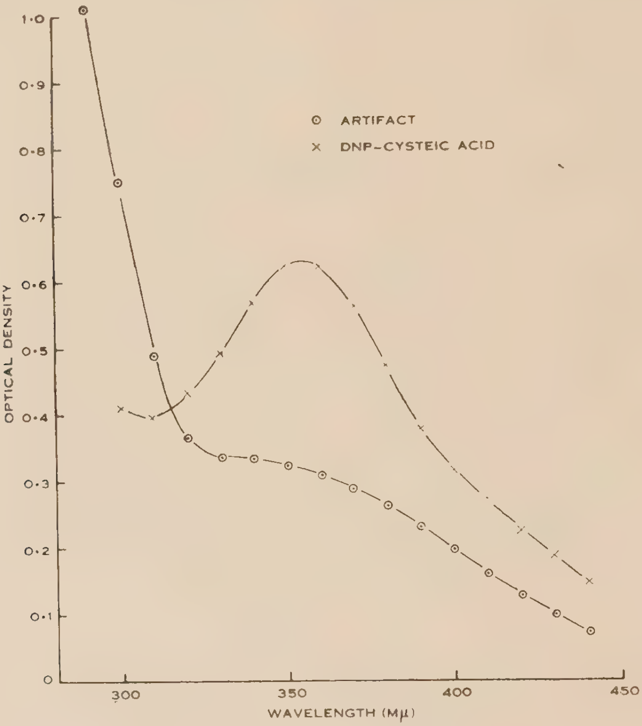
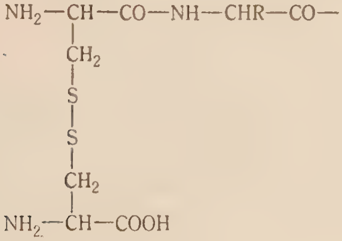


Fig. 1.—Absorption curves of DNP-cysteic acid (×) and of the yellow artifact (⊙) isolated from hydrolysates of oxidized **DNP-wool**. The optical densities are for the extract from 105 mg DNP-wool in 4 ml of 1 per cent. $NaHCO_3$.

V. DISCUSSION

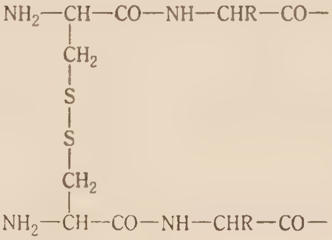
For the detection and estimation of *N*-terminal cystine or half-cystine residues in wool, oxidation has been preferred to reduction (followed by alkylation) since the oxidation is irreversible whereas reduction with thiols is reversible and special conditions are required to ensure complete reduction of all disulphide bonds. Incorrect results would arise from experiments in which reduction was not complete since *N*-terminal half-cystine residues could be included in the non-reduced cystine fraction and hence not detectable as a stabilized DNP- or PTH-derivative. Moreover the high concentrations of urea (Lindley 1955), guanidine (Katchalski, Benjamin, and Gross 1957) or detergents (Markus and Karush 1957) necessary to facilitate complete reduction of disulphide bonds in proteins makes the purification of the fully reduced and alkylated protein difficult since it is essential that no loss of small fragments takes place. This could occur, for example, in dialysis. Such fragments could be released following scission of the –S–S– bonds binding them to the main protein chain. Thus attempts by the author to detect any *N*-terminal half-cystine residues in bovine plasma albumin which had been reduced for 16 hr with thioglycollate at pH 8 in the presence of 8M urea and then alkylated with iodoacetic acid gave equivocal results. Analyses showed that sufficient cystine was present for one to two disulphide bonds per molecule, which could include non-reduced *N*-terminal half-cystine residues if present. Moreover, although only one equivalent of phenylthiohydantoins (the *N*-terminal aspartic acid residue) was estimated by the Edman (1950) method on the protein, both before and after the reduction and alkylation treatment, the removal of reagents by dialysis could have resulted in the loss of a small fragment with *N*-terminal *S*-carboxymethylcysteine residues. Oxidation experiments (Thompson 1958), in which excess reagent was removed by freeze-drying, showed that no *N*-terminal half-cystine residues are present in this protein.

The detection of DNP-cysteic acid in hydrolysates of oxidized DNP-wool shows that terminal residues of half-cystine or cystine are present with a free α-amino group. It is not possible from the data to say whether both these groups or only one of them is present. Acetone extraction of oxidized DNP-wool before hydrolysis gave no detectable trace of DNP-cysteic acid. This suggests that *N*-terminal cystine with only one of the half-cystine carboxyls in peptide linkage (I) is not present in wool, but the possibility of *N*-terminal cystine with both carboxyl groups bound in peptide chains (II) is possible. *N*-terminal half-cystine residues could be linked by the –S–S– bond to the other half-cystine residue either intramolecularly (III) as is the case in oxytocin, vasopressin, and insulin (Anfinsen and Redfield 1956) or intermolecularly (IV). The method used would not detect *N*-terminal cysteine residues if present in wool since dinitrophenylation would form *N*, *S*-bis-dinitrophenylcysteinyl residues which would not give DNP-cysteic acid on oxidation.



$$\begin{array}{lcl}
\mathrm{NH_2}- & \mathrm{CH} & -\mathrm{CO-NH-CHR-CO-}\\
 & | & \\
 & \mathrm{CH_2} & \\
 & | & \\
 & \mathrm{S} & \\
 & | & \\
 & \mathrm{S} & \\
 & | & \\
 & \mathrm{CH_2} & \\
 & | & \\
\mathrm{NH_2}- & \mathrm{CH} & -\mathrm{COOH}
\end{array}$$

(I)

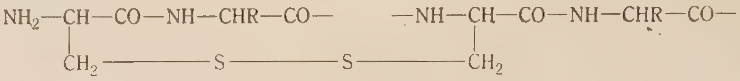


$$\begin{array}{lcl}
\mathrm{NH_2}- & \mathrm{CH} & -\mathrm{CO-NH-CHR-CO-}\\
 & | & \\
 & \mathrm{CH_2} & \\
 & | & \\
 & \mathrm{S} & \\
 & | & \\
 & \mathrm{S} & \\
 & | & \\
 & \mathrm{CH_2} & \\
 & | & \\
\mathrm{NH_2}- & \mathrm{CH} & -\mathrm{CO-NH-CHR-CO-}
\end{array}$$

(II)

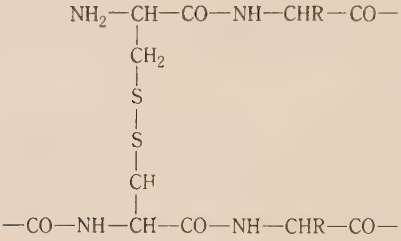
The number of different *N*-terminal residues in wool is therefore at least eight, comprising aspartic and glutamic acids, serine, threonine, glycine, alanine, valine, and cystine or half-cystine. Quantitatively, terminal half-cystine or cystine groups form the second most abundant of these. It must be remembered, however, that the correction factors for the destruction of DNP-glycine, the most abundant terminal amino acid, and DNP-cysteic acid (Table 2) are considerably higher than those for the other DNP-amino acids isolated (Thompson 1957) and consequently less confidence can be placed on these values.

The total free α-amino groups in wool, previously thought to be 20 μmoles per g (Thompson 1957, including a summary of the results of other workers), must now be raised to 28 μmoles per g.



$$\begin{array}{lclcl} NH_2- & CH & -CO-NH-CHR-CO- \qquad -NH- & CH & -CO-NH-CHR-CO- \\ & | & & | & \\ & CH_2 & -S-S- & CH_2 & \end{array}$$

(III)



$$\begin{array}{rcl} NH_2- & CH & -CO-NH-CHR-CO- \\ & | & \\ & CH_2 & \\ & | & \\ & S & \\ & | & \\ & S & \\ & | & \\ & CH & \\ & | & \\ -CO-NH- & CH & -CO-NH-CHR-CO- \end{array}$$

(IV)

The difficulty in interpreting the significance of the quantitative results for *N*-terminal groups in a protein as histologically complex as the wool fibre has previously been discussed by the author (Thompson 1957). The detection of an additional *N*-terminal group invalidates the interpretation in terms of a molecular model along the lines suggested by Middlebrook (1951). Calculations of average chain weights from the *N*-terminal groups (Middlebrook 1951; Alexander and Smith 1956), which are of limited significance with polypeptide chains of varying size, are meaningless if all the terminal groups are not measured or if cyclic molecules are present. The detection of *N*-terminal cystine or half-cystine residues in wool has not increased the number of terminal groups sufficiently to make untenable the previous suggestion that wool is predominantly a collection of cyclic molecules held together by disulphide linkages (Thompson 1957). Alternatively, masked *N*-terminal (Narita 1958) and *C*-terminal groups may be present.

VI. ACKNOWLEDGMENT

The author wishes to thank Mr. W. B. Hall for the statistical analysis.

VII. REFERENCES

ALEXANDER, P., HUDSON, R.F., and FOX, M. (1950).—*Biochem. J.* **46**: 27.

ALEXANDER, P., and SMITH, L. F. (1956).—Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. B. p. B-56.

ANFINSEN, C. B., and REDFIELD, R. R. (1956).—*Advanc. Protein Chem.* **11**: 1.

ANFINSEN, C. B., SELA, M., and TRITCH, H. (1956).—*Arch. Biochem. Biophys.* **65**: 156.

BETTELHEIM, F. R. (1955).—*J. Biol. Chem.* **212**: 235.

DURRUM, E. L. (1951).—*J. Colloid Sci.* **6**: 274.

EDMAN, P. (1950).—*Acta Chem. Scand.* **4**: 283.

EDMAN, P., and LAUBER, K. (1956).—*Acta Chem. Scand.* **10**: 466.

FRITZE, E. R., and ZAHN, H. (1956).—Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. C. p. C-119.

KATCHALSKI, E., BENJAMIN, G. S., and GROSS, V. (1957).—*J. Amer. Chem. Soc.* **79**: 4096.

LEVY, A. L. (1954).—*Nature* **174**: 126.

LINDLEY, H. (1955).—*J. Amer. Chem. Soc.* **77**: 4927.

MARKUS, G., and KARUSH, F. (1957).—*J. Amer. Chem. Soc.* **79**: 134.

MIDDLEBROOK, W. R. (1951).—*Biochim. Biophys. Acta* **7**: 547.

NARITA, K. (1958).—*Biochim. Biophys. Acta* **28**: 184.

PORTER, R. R., and SANGER, F. (1948).—*Biochem. J.* **42**: 287.

SANGER, F. (1945).—*Biochem. J.* **39**: 507.

SANGER, F. (1949).—*Biochem. J.* **45**: 563.

THOMPSON, E. O. P. (1954).—*J. Biol. Chem.* **207**: 563.

THOMPSON, E. O. P. (1957).—*Aust. J. Biol. Sci.* **10**: 225.

THOMPSON, E. O. P. (1958).—*Biochim. Biophys. Acta* **29**: 643.

THOMPSON, E. O. P., and O'DONNELL, I. J. (1959).—*Aust. J. Biol. Sci.* **12**: 282.

TITANI, K., YOSHIKAWA, H., and SATAKE, K. (1956).—*J. Biochem., Tokyo* **43**: 737.

THE DEVELOPMENT AND GROWTH OF VIBRISSAE IN THE HOUSE MOUSE WITH PARTICULAR REFERENCE TO THE TIME OF ACTION OF THE TABBY (*Ta*) AND RAGGED (*Ra*) GENES

By R. B. Dun*

[*Manuscript received January* 13, 1959]

Summary

The order of development of certain vibrissae, found on the head and fore limbs of the mouse, has been determined using observations on the developmental stage and relative length of each follicle at 11, 12, 13, and 14 days foetal age. The length of vibrissal fibres at birth was also utilized.

In homozygous ragged (*RaRa*) mice, no vibrissa follicle commencing development after approximately 12 days foetal age produces a functional fibre. This is in contrast to the tabby genotypes where there is no precisely timed gene action, but within any group of vibrissae the incidence follows the order of follicle initiation.

I. Introduction

Only three of the genes known in the mouse which affect the development of the skin and hair influence the production of specific types of hair. The genes which fall into this classification are crinkled (*cr*) (Falconer, Fraser, and King 1951), tabby (*Ta*) (Falconer 1953), a sex-linked mimic of crinkled, and ragged (*Ra*) (Carter and Phillips 1954). Because of their specific effects, they furnish favourable material for detecting the onset of gene action, provided the times at which each type of follicle commences development are known.

Dry (1926) classified the coat of the mouse into the following hair types:

(1) Tactile hairs or vibrissae
(2) Hairs of the coat proper
  (*a*) Guard hairs
  (*b*) Awls
  (*c*) Auchenes
  (*d*) Zig-zags

Considering the vibrissae first, Figure 1 shows the distribution of the more prominent vibrissae of the mouse.

Certain mystacial vibrissae are the first follicles to commence growth. Grüneberg (1943*a*) states that, in the $12\frac{1}{2}$-day embryo, three rows of mystacial follicles were present and that the full complement of rows was not seen until $13\frac{1}{2}$ days. Davidson and Hardy (1952) examined the stages in the development of vibrissae using one or more of the most posterior mystacials which were observed to commence growth at 12 days. Because of the difficulty in orientating the mystacial

---

* New South Wales Department of Agriculture, Agricultural Experiment Station, Trangie, N.S.W.

follicles, no information is available comparing the time of initiation of individual vibrissae.

More information is available comparing the development of certain members of the minor groups of vibrissae. Grüneberg (1943*a*) says that in the $12\frac{1}{2}$-day mouse embryo, one supra-orbital follicle is always present and that the postorbital follicle is sometimes visible. At $13\frac{1}{2}$ days, the supra-orbitals, postorbital, and postoral vibrissae were all observed. No other sites were examined.

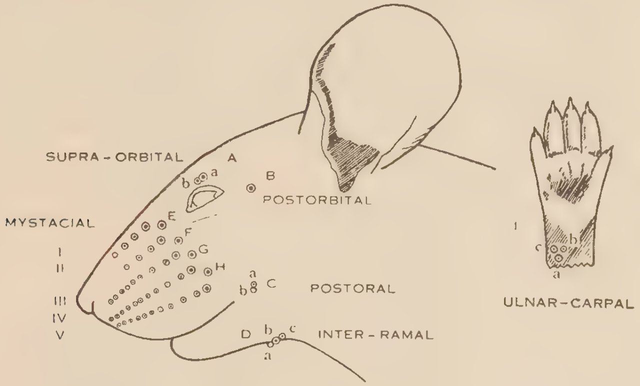


Fig. 1.—Head and distal part of the right fore limb of the mouse showing distribution of vibrissae.

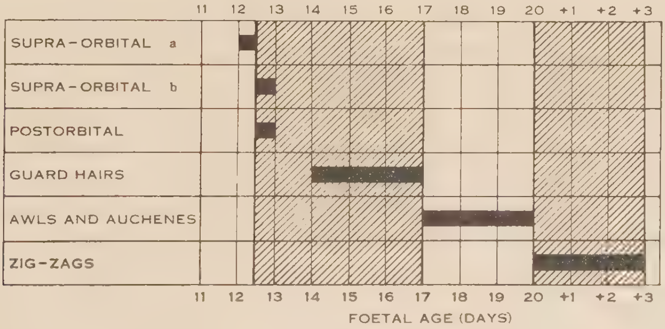


Fig. 2.—Action of the crinkled gene related to the time of initiation of vibrissae and pelage-hair types in the mouse (after Falconer, Fraser, and King (1951).

Falconer, Fraser, and King (1951) examined the supra-orbital and postorbital sites in their analysis of the crinkled gene. At $12\frac{1}{2}$ days, one supra-orbital follicle was well developed and by $13\frac{1}{2}$ days the second follicle was present. The postorbital vibrissa had just commenced development in the $12\frac{1}{2}$-day foetus and was well developed by $13\frac{1}{2}$ days. The postoral follicles were present at $13\frac{1}{2}$ days but it was impossible to distinguish any details of follicle morphology. These authors found the remaining vibrissae too difficult to examine in their material.

With regard to the coat hairs, Falconer, Fraser, and King (1951), after examining sagittal sections of skin from the mid-dorsum of the body, suggested that the type of fibre was related to the age at which the follicles were formed. The order of development would be guard hairs first, then awls and auchenes, and lastly the zig-zags. They proposed a model for the action of the crinkled gene which is illustrated in Figure 2. The black bands show the periods during which the follicles of each type are commencing development, while the cross-hatched areas indicate the periods when the initiation of follicles is stopped by the crinkled gene. Thus the coat of the crinkled mouse lacks certain vibrissae and all guard hairs and zig-zag fibres. Falconer, Fraser, and King (1951) showed that the coat of the crinkled mouse possessed one type of fibre, the follicles of which had commenced growth between 17 and 20 days gestation. In length these fibres resembled awls but their internal structure was not characteristic.

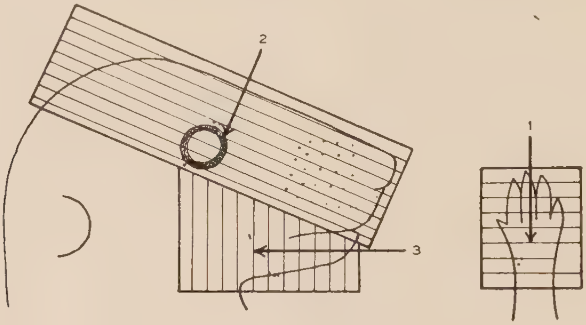


Fig. 3.—Sectioning planes for examining the development of specific vibrissa follicles.

One of the main difficulties with this model is that there is insufficient knowledge of the development of individual vibrissa follicles. If the development of all follicles which are normally initiated between $12\frac{1}{2}$ and 17 days is inhibited by the crinkled gene, it is difficult to understand why some mystacial vibrissae are not affected. As this seemed to be a crucial point in the theory, it was decided to examine the time of initiation of many vibrissae and relate this information to the vibrissae grown by tabby mice. The sex-linked tabby was used in preference to its autosomal mimic crinkled, as tabby is the gene being used in a selection experiment which is being conducted at this Laboratory (Dun and Fraser 1958). Observations were also made on the vibrissae of ragged mice to see if there was any similarity with the tabby–crinkled syndrome.

II. Materials and Methods

Foetuses of known ages were obtained by recording the date at which vaginal plugs were observed in a mating between inbred lines (CBA males × Aw101 females). Litters were obtained at $11\frac{1}{2}$, $12\frac{1}{2}$, $13\frac{1}{2}$, and $14\frac{1}{2}$ days gestation and the calculated ages were checked by examining the external features of the embryos (Grüneberg 1943*b*).

TABLE 1

PERCENTAGE INCIDENCE OF VIBRISSAE IN THE MINOR GROUPS OF MICE WITH TABBY AND NORMAL GENOTYPES

Vibrissae scored on both sides of the head

Genotype	Number of Mice	Supra-orbital		Post-orbital	Postoral		Inter-ramal			Ulnar–Carpal		
		a	*b*		*a*	*b*	*a*	*b*	*c*	*a*	*b*	*c*
+·	224	100·0	99·6	100·0	100·0	100·0	95·5	100·0	95·5	100·0	99·6	100·0
++	230	100·0	100·0	100·0	100·0	99·6	95·2	100·0	95·2	100·0	99·6	100·0
Ta+	227	100·0	96·5	92·7	99·3	15·0	34·6	100·0	34·6	100·0	72·2	98·5
Ta·	256	100·0	56·8	1·6	75·4	0	8·2	100·0	8·2	100·0	2·7	45·9

Two litters of five mice each were obtained at the different ages and fixed in Bouin's fluid. After embedding the foetuses in paraffin wax, serial sections (8 μ thick) were stained with haematoxylin and eosin. Serial sections were made of the head and fore legs (Fig. 3) to allow histological examination of the vibrissae.

TABLE 2

NUMBER OF VIBRISSAE IN THE ROWS OF THE MYSTACIAL GROUPS OF MICE WITH TABBY AND NORMAL GENOTYPES

Genotype	Number of Mice	Row E–F–G–H	Row I	Row II	Row III	Row IV	Row V
+ ·	84	4·00	3·98	4·00	9·75	10·63	11·67
++	84	4·00	4·00	3·99	9·72	10·70	11·57
Ta+	96	4·00	3·94	3·83	9·48	10·73	11·38
Ta ·	110	4·00	3·92	3·90	9·25	10·84	11·22

During microscopic examination of the serial sections, scale drawings of each vibrissae follicle were made using a measuring eyepiece. The stage of growth of each follicle was recorded using the numerical scores of Davidson and Hardy (1952).

TABLE 3

DIFFERENCES BETWEEN TABBY GENOTYPES IN THE NUMBER OF VIBRISSAE IN MYSTACIAL ROWS III, IV, AND V

Genotypes	Row III		Row IV		Row V	
	Difference	*t*	Difference	*t*	Difference	*t*
++ minus *Ta*+	+0·24	2·35*	−0·03	0·26	+0·19	1·98*
+ · minus *Ta* ·	+0·50	4·67**	−0·21	1·78	+0·45	3·85**
Ta+ minus *Ta* ·	+0·23	2·32*	−0·11	0·96	+0·16	1·50

$^{*}P < 0{\cdot}05$. $^{**}P < 0{\cdot}01$.

With the 13½- and 14½-day foetuses, it was not possible to examine mystacial rows I and II in this way as the follicles had been sectioned obliquely. When examining the sectioned material, difficulty was encountered in that, of 10 foetuses prepared at each age, very few were found to be suitable for microscopy. In many cases follicles could not be measured with certainty because of oblique sections or imperfect preparation. This meant that no estimates of the variation between individuals

TABLE 4

PERCENTAGE INCIDENCE OF VIBRISSAE IN THE MINOR GROUPS OF MICE WITH RAGGED AND NORMAL GENOTYPES

Genotype	Number of Mice	Supra-orbital		Post-	Postoral		Inter-ramal			Ulnar-Carpal		
		a	*b*	orbital	*a*	*b*	*a*	*b*	*c*	*a*	*b*	*c*
++	35	100·0	100·0	100·0	100·0	100·0	88·3	100·0	88·3	100·0	100·0	100·0
Ra+	60	100·0	100·0	100·0	100·0	100·0	91·5	100·0	91·5	100·0	100·0	100·0
RaRa	32	90·6	0	75·0	0	0	0	0	0	0	0	0

could be made. The values quoted refer only to means of the two or three foetuses of each age which could be adequately measured.

The presence of the minor groups of vibrissae on living mice was scored by naked eye using a desk lamp. Two scores were made, the first at 5 days and a check score 10 days after birth. Vibrissae were scored on both sides of the head. The vibrissae in the mystacial rows were counted in new-born mice using a dissection microscope. In the case of the homozygous ragged mice, the mystacial vibrissae were scored at 10 days of age but, if death occurred prior to this time, the vibrissae were still readily counted in mice more than 5 days old. The mice of the various tabby and ragged genotypes examined were the F_2 of CBA-based stocks mated to Aw101.

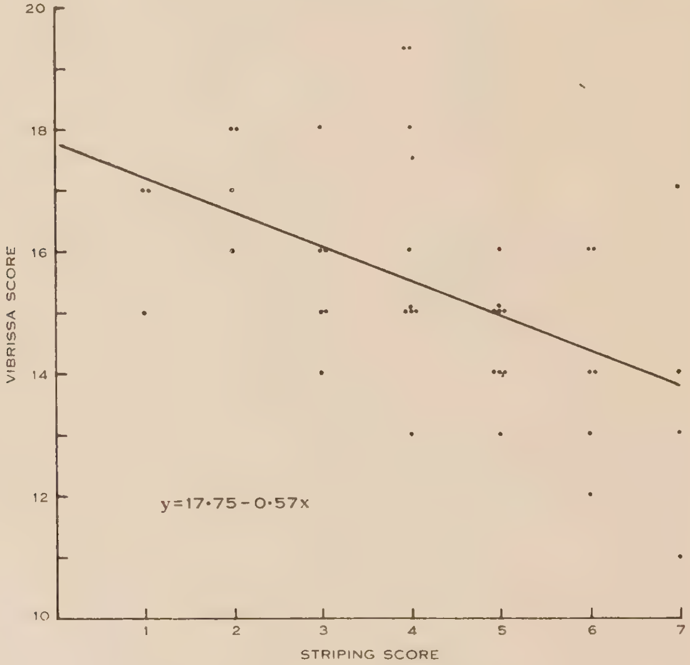


Fig. 4.—Influence of the degree of striping of the coat on vibrissa number in heterozygous tabby female mice.

The vibrissae of tabby females (*Ta*+) were examined and their coats were seen to vary from near normality through various intensities of striping to a type which was almost indistinguishable from that of the tabby hemizygous male (*Ta*·). The tabby females were scored for degree of striping at 45 days of age using a series of six end-point photographs. Thus scores ranged from 1 to 7, 1 being for the least degree of striping.

In order to measure the effect of the tabby gene on the growth of vibrissae, matings were arranged between normal males (+·) and tabby females (*Ta*+). The litters were examined at birth and only those which contained a member of each of the four genotypes (+·, *Ta*·, ++, and *Ta*+) were included in the experiment. The litters were standardized to four mice as above and the lengths of vibrissae were measured at day 1, 5, 9, and 21. Five litters remained intact until the final measurement and provided the data for the estimation of growth rates. Only the supra-orbital, postorbital, postoral, and inter-ramal groups were measured throughout but, at day 1, the ulnar–carpal group and vibrissa G and row IV mystacials were measured in the normal mice.

III. Results

The incidence of the vibrissae in the minor groups of tabby females, tabby males, and wild-type mice are shown in Table 1. The vibrissae which were missing from the inter-ramal group were presumed to be equal numbers of either *a* or *c*. With the exception of the inter-ramals, all vibrissae are almost invariably present in wild-type mice. Many vibrissae are missing in tabby mice, the effect being more marked in the hemizygous male than in the heterozygous female.

Table 5

NUMBER OF VIBRISSAE IN THE ROWS OF THE MYSTACIAL GROUP IN MICE WITH RAGGED AND NORMAL GENOTYPES

Genotype	Row E–F–G–H	Row I	Row II	Row III	Row IV	Row V
++	4·00	4·00	4·00	9·70	10·68	11·72
Ra+	4·00	4·00	4·00	9·69	10·60	11·72
RaRa	4·00	0·94	0·97	1·28	0·97	0·50

The relationship between the total number of vibrissae present in the minor groups and the degree of striping on the coats of tabby females, is shown in Figure 4. There was a highly significant negative correlation of −0·53 between striping score and vibrissae number. The repeatability of the striping score was 0·87.

Table 2 shows the number of vibrissae in the single vertical and the five horizontal rows which comprise the mystacial group. The significance of the differences between horizontal rows III, IV, and V for the different genotypes are shown in Table 3.

The vertical row E–F–G–H and mystacial rows I and II normally have four vibrissae each. This arrangement is hardly affected by the tabby gene although there tends to be slightly more mice with *d*, the most anterior vibrissa, missing from rows I and II. Rows III, IV, and V have much more variable counts in normal mice. Tables 2 and 3 show that rows III and V have slightly but significantly less numbers of vibrissae in tabby mice. Row IV shows no significant differences. The actual vibrissae which may be missing in rows III and V of tabby mice could not be identified as there was a continuous gradation in length of vibrissae down the rows and there were no large gaps between vibrissae. Thus, the tabby gene has little effect on the number of mystacial vibrissae.

TABLE 6

GROWTH OF CERTAIN VIBRISSAE IN TABBY AND NORMAL MICE FROM DAY 1 TO 21 DAYS OF AGE

Genotype	Vibrissa	Day 1		Day 5		Day 9		Day 21	
		Length (mm)	Variance	Length (mm)	Variance	Length (mm)	Variance	Length (mm)	Variance
+·	Supra-orbital *a*	1·53	0·712	3·84	3·004	6·66	6·360	13·00	3·333
	Postorbital	1·07	0·201	2·95	1·250	5·74	4·471	13·00	4·444
	Postoral *a*	0·46	0·160	1·64	0·404	3·57	0·779	6·50	1·667
	Inter-ramal *b*	0·34	0·130	1·26	0·330	2·70	0·650	6·40	1·750
++	Supra-orbital *a*	1·59	0·277	3·97	1·690	6·77	1·401	12·70	1·778
	Postorbital	1·07	0·246	3·09	0·699	5·82	0·996	13·15	5·580
	Postoral *a*	0·52	0·151	1·67	0·246	3·88	0·751	6·60	1·556
	Inter-ramal *b*	0·36	0·030	1·40	0·100	2·76	1·030	6·60	4·250
Ta+	Supra-orbital *a*	1·33	0·312	3·44	1·004	6·19	3·299	11·55	4·694
	Postorbital	0·85	0·049	2·69	1·677	5·06	1·782	11·75	0·694
	Postoral *a*	0·28	0·317	1·10	0·054	2·71	0·432	5·50	1·667
	Inter-ramal *b*	0·33	0·018	1·16	1·180	2·52	1·070	5·70	0·750
Ta·	Supra-orbital *a*	1·18	0·996	3·10	2·400	5·98	7·440	10·25	9·583
	Postorbital	—	—	0·52	0·084	1·56	1·027	3·70	1·778
	Postoral *a*	0·18	0·062	0·86	0·360	2·08	0·262	4·77	4·068
	Inter-ramal *b*	0·24	0·040	1·10	0·300	2·32	0·870	6·20	5·750

TABLE 7

LINEAR REGRESSION EQUATIONS DESCRIBING THE GROWTH OF CERTAIN VIBRISSAE IN MICE WITH TABBY AND NORMAL GENOYTPES

x = age in days; y = length of vibrissae (mm)

Vibrissa	+·		++		*Ta*+		*Ta*·	
	Regression Equation	Fiducial Limits of Constant	Regression Equation	Fiducial Limits of Constant	Regression Equation	Fiducial Limits of Constant	Regression Equation	Fiducial Limits of Constant
Supra-orbital *a*	$y = 0 \cdot 573x + 1 \cdot 101$	±0·026	$y = 0 \cdot 553x + 1 \cdot 281$	±0·020	$y = 0 \cdot 510x + 1 \cdot 038$	±0·024	$y = 0 \cdot 452x + 1 \cdot 062$	±0·036
Postorbital	$y = 0 \cdot 606x + 0 \cdot 236$	±0·024	$y = 0 \cdot 611x + 0 \cdot 284$	±0·020	$y = 0 \cdot 551x + 0 \cdot 129$	±0·014	$y = 0 \cdot 194x - 0 \cdot 337$	±0·018
Postoral *a*	$y = 0 \cdot 303x + 0 \cdot 316$	±0·018	$y = 0 \cdot 305x + 0 \cdot 348$	±0·016	$y = 0 \cdot 265x + 0 \cdot 015$	±0·012	$y = 0 \cdot 234x - 0 \cdot 133$	±0·014
Inter-ramal *b*	$y = 0 \cdot 308x - 0 \cdot 097$	±0·024	$y = 0 \cdot 316x - 0 \cdot 064$	±0·022	$y = 0 \cdot 274x - 0 \cdot 046$	±0·014	$y = 0 \cdot 304x - 0 \cdot 271$	±0·026

Tables 4 and 5 show the vibrissae which are present in mice with different ragged genotypes. The heterozygous ragged mice (*Ra*+) had a complement of vibrissae which was indistinguishable in number from that of wild-type, but when mice were homozygous the only vibrissa follicles which produced fibres were the most posterior mystacials and the two largest vibrissae in the minor groups. Follicle growth was observed at the sites of certain other vibrissae but in no case did a fibre emerge. The comparison between tabby hemizygous males and homozygous ragged mice is illustrated in Figure 5.

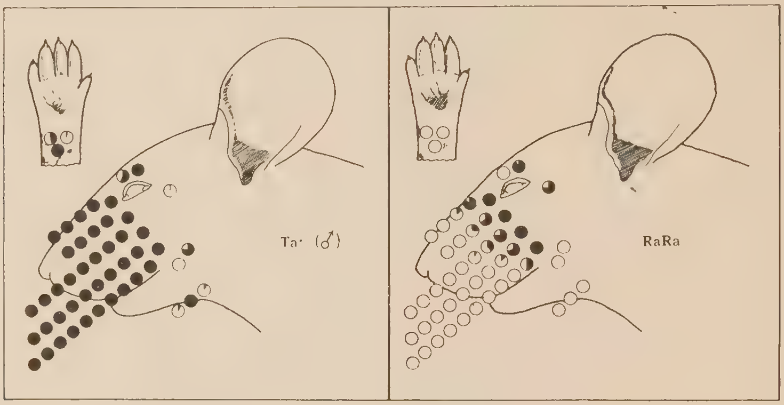


Fig. 5.—Incidence of vibrissae in tabby males and homozygous ragged mice. The percentage incidence of each vibrissa is shown as the proportion (black shading) of a circle (indicating a vibrissal site).

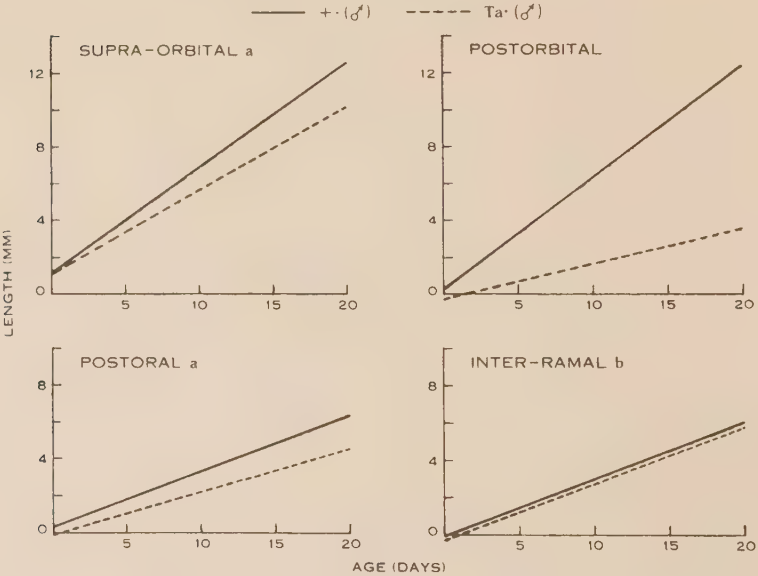


Fig. 6.—Comparisons in growth rate between vibrissae of normal male and tabby male mice.

The growth in fibre length of certain vibrissae was examined in mice with normal and tabby genotypes. These results are shown in Tables 6 and 7. In general, the tabby gene exerts a depressant effect on the length of vibrissae at birth and on the subsequent growth rate. As with other characteristics, the tabby female tends to be intermediate between the wild type and the tabby male. Figure 6 demonstrates that the reduction in the growth rate of vibrissae is much more marked at certain sites, e.g. postorbital than at others, e.g. inter-ramal *b*.

Table 1 shows that a typical postorbital vibrissa is rarely recorded in tabby males. However, a fibre which cannot be demonstrated at birth is invariably seen at the postorbital site at 5 days of age. This fibre shows slow growth as shown in Tables 6 and 7 and Figure 6 but it is atypical in that it reaches a length about a third that of the normal and it tends to curl into and be hidden by the hairs of the coat.

TABLE 8

AVERAGE LENGTH OF VIBRISSAE AT BIRTH IN NORMAL MICE

Measurements are means for 10 mice

Vibrissa	Length (mm)	Vibrissa	Length (mm)
Supra-orbital *a*	1·56	Mystacial G	2·18
Supra-orbital *b*	0·75	Row IV *a*	1·71
Postorbital	1·07	,, *b*	1·33
Postoral *a*	0·49	,, *c*	0·96
Postoral *b*	0·38	,, *d*	0·73
Inter-ramal *a*	0·24	,, *e*	0·45
Inter-ramal *b*	0·35	,, *f*	0·32
Inter-ramal *c*	0·22	,, *g*	0·21
Ulnar–carpal *a*	0·18	,, *h*	0·20
Ulnar–carpal *b*	0·05	,, *i*	0·20
Ulnar–carpal *c*	0·14	,, *j*	0·19

"Vestigial vibrissae" were suspected of being present at certain other sites in tabby males, e.g. supra-orbital *b* and postoral *b*. Many such cases were recorded at 5 days but the fibres could not be distinguished when making subsequent measurements.

Table 8 shows the average length of the vibrissae which were measured at day 1 in normal mice. Tables 9 and 10 and Figures 7 and 8 show the stages of development and lengths of vibrissae follicles in 12½-, 13½-, and 14½-day normal embryos. There was no sign of follicle development in the 11½-day foetuses. With the mystacial vibrissae, the only results listed are for vibrissa *G* and row IV as longitudinal sections of these follicles were obtained at all ages.

The stages of development reached by all follicles at 12½ days foetal age are shown in Figure 9. In this case it was possible to examine approximately longitudinal sections of all mystacial vibrissae because the largest follicle was only at the pre-papilla stage.

Table 9

STAGES OF DEVELOPMENT AND LENGTHS OF FOLLICLES FOR THE MINOR GROUPS OF VIBRISSAE IN 12-, 13-, AND 14-DAY NORMAL FOETUSES

Vibrissa	12½-day Foetus*		13½-day Foetus†		14½-day Foetus†	
	Developmental Stage	Length of Follicle (mm)	Developmental Stage	Length of Follicle (mm)	Developmental Stage	Length of Follicle (mm)
Supra-orbital *a*	1	0·052	3*b*	0·256	5	0·500
Supra-orbital *b*	1	0·024	2	0·100	3*c*	0·260
Postorbital	1	0·040	3*a*	0·172	4	0·328
Postoral *a*	—	—	2	0·080	3*b*	0·220
Postoral *b*	—	—	2	0·088	3*b*	0·220
Inter-ramal *a*	—	—	M.P.‡	—	2	0·104
Inter-ramal *b*	—	—	M.P.	—	3*a*	0·132
Inter-ramal *c*	—	—	M.P.	—	2	0·116
Ulnar–carpal *a*	—	—	M.P.	—	2	0·112
Ulnar–carpal *b*	—	—	M.P.	—	1	0·052
Ulnar–carpal *c*	—	—	M.P.	—	1	0·068

* Three foetuses from one litter measured.

† Two foetuses from one litter measured.

‡ Mesodermal proliferation.

Table 10

STAGES OF DEVELOPMENT AND LENGTH OF FOLLICLES FOR CERTAIN MYSTACIAL VIBRISSAE IN 12-, 13-, AND 14-DAY NORMAL FOETUSES

Vibrissa	12½-day Foetus*		13½-day Foetus†		14½-day Foetus†	
	Developmental Stage	Length of Follicle (mm)	Developmental Stage	Length of Follicle (mm)	Developmental Stage	Length of Follicle (mm)
Mystacial G	2	0·088	4	0·360	5	0·664
Row IV *a*	1	0·056	3*c*	0·300	5	0·568
,, *b*	1	0·036	3*b*	0·220	5	0·468
,, *c*	1	0·028	3*a*	0·172	4	0·392
,, *d*	—	—	3*a*	0·116	4	0·332
,, *e*	—	—	2	0·076	3*c*	0·264
,, *f*	—	—	1	0·048	3*b*	0·192
,, *g*	—	—	1	0·036	3*a*	0·140
,, *h*	—	—	—	—	2	0·092
,, *i*	—	—	—	—	2	0·072
,, *j*	—	—	—	—	1	0·056
,, *k*	—	—	—	—	1	0·044
,, *l*	—	—	—	—	1	0·032

* Three foetuses from one litter measured.

† Two foetuses from one litter measured.

Although the present data is insufficient to allot an accurate developmental time to each vibrissa follicle, it is possible to arrange the vibrissae in the order in which they commence development. It has been assumed that the size and stage of development of a follicle were indications of its age. Use was also made of the relationship between follicle length at 14½ days and the length of the tactile hairs at birth ($r = 0{\cdot}96$) when deciding between members of a group which commence development almost simultaneously. For example, postoral *a* and *b* could not be differentiated on histological examination but length measurements at birth showed

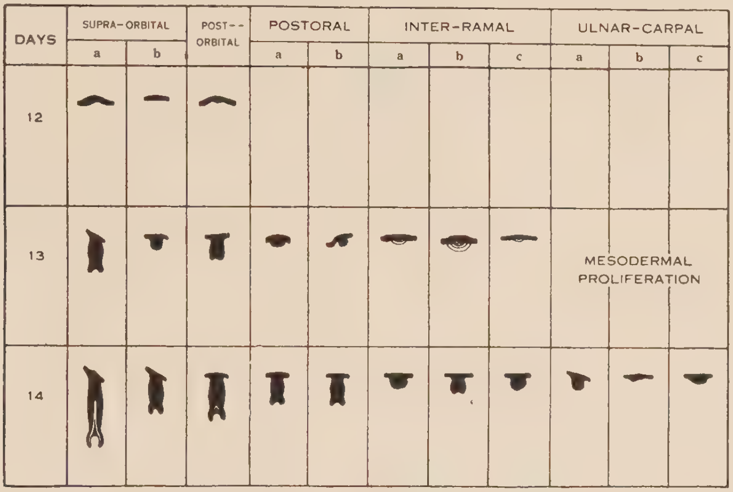


Fig. 7.—Longitudinal sections of the developing follicles in the minor groups of vibrissae at 12½, 13½, and 14½ days foetal age in normal mice.

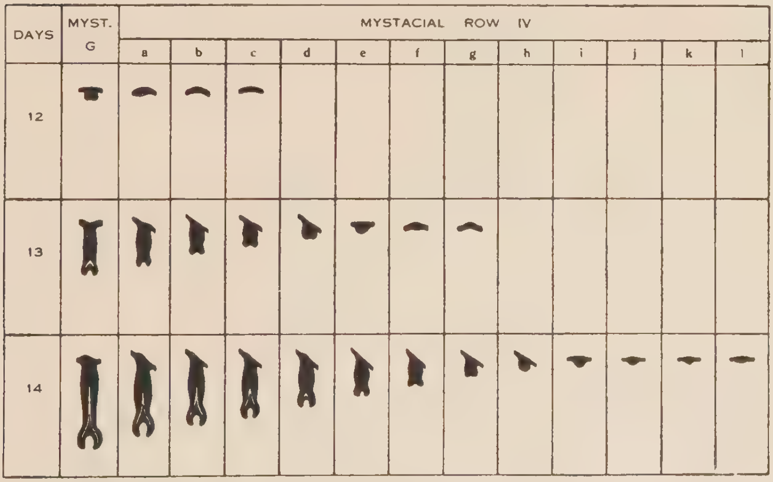


Fig. 8.—Longitudinal sections of the developing follicles of certain mystacial vibrissae at 12½, 13½, and 14½ days foetal age in normal mice.

that the dorsal member of the pair, postoral *a*, was slightly but consistently longer than *b*. This indicated that *a* preceded *b* very slightly in time of initiation. Table 11 therefore shows the order of initiation of the vibrissae, the measurements used to form the list, and the percentage incidence of each vibrissa in tabby males and homozygous ragged mice.

TABLE 11

CERTAIN VIBRISSAE OF THE MOUSE ARRANGED IN THE ORDER IN WHICH THEY COMMENCE DEVELOPMENT

Some results from Tables 1, 2, 4, 9, and 10 are included to illustrate why this sequence was chosen and to show the incidence of each vibrissa in tabby male and homozygous ragged mice

Vibrissa	Length (mm) of Follicles at:				Percentage Incidence of Vibrissae in:	
	12 Days	13 Days	14 Days	Birth	Tabby Males	Homozygous Ragged Mice
1. Mystacial G	0·088	0·360	0·664	2·18	100·0	100·0
2. Mystacial row IV *a*	0·056	0·300	0·568	1·71	100·0	93·7
3. Supra-orbital *a*	0·052	0·256	0·500	1·56	100·0	91·0
4. Postorbital	0·040	0·172	0·328	1·07	1·6	75·0
5. Mystacial row IV *b*	0·036	0·220	0·468	1·33	100·0	3·1
6. Mystacial row IV *c*	0·028	0·172	0·392	0·96	100·0	0
7. Supra-orbital *b*	0·020	0·100	0·260	0·75	56·8	0
8. Mystacial row IV *d*	—	0·116	0·332	0·73	100·0	0
9. Postoral *a*	—	0·080	0·220	0·49	75·4	0
10. Mystacial row IV *e*	—	0·076	0·264	0·45	100·0	0
11. Postoral *b*	—	0·088	0·220	0·38	0	0
12. Mystacial row IV *f*	—	0·048	0·192	0·32	100·0	0
13. Mystacial row IV *g*	—	0·036	0·140	0·21	100·0	0
14. Inter-ramal *b*	—	—	0·132	0·35	100·0	0
15, 16. Inter-ramals *a* and *c*	—	—	0·112	0·23	8·2	0
17. Ulnar–carpal	—	—	0·112	0·18	100·0	0
18. Mystacial row IV *h*	—	—	0·092	0·20	100·0	0
19. Mystacial row IV *i*	—	—	0·072	0·20	100·0	0
20. Ulnar–carpal *c*	—	—	0·068	0·14	45·9	0
21. Mystacial row IV *j*	—	—	0·056	0·19	100·0	0
22. Ulnar–carpal *b*	—	—	0·052	0·05	2·7	0
23. Mystacial row IV *k*	—	—	0·044	0·19	100·0	0
24. Mystacial row IV *l*	—	—	0·032	0·19	100·0	0

IV. DISCUSSION

The difference in the times of action of the two genes is very striking. In the case of the homozygous ragged mouse, no follicle commencing after 12½ days foetal age produces a functional fibre with the result that the body is almost completely naked, possessing only those fibres produced by the 12½-day vibrissae (note the almost perfect analogy between the incidence of vibrissae in the *RaRa* mouse (Fig. 5) and the follicles of the 12½-day normal foetus (Fig. 9)). Slee (1957) has shown that the skin of homozygous ragged mice has a lowered density of follicles which rarely carry hairs. Our results show that hair eruption occurs only in those follicles which commence growth before 12½ days foetal age.

On the other hand, tabby does not have a precisely timed gene action. The two most outstanding illustrations of this fact are listed below:

(1) The mystacial vibrissae which are initiated in continuous sequence between 12 and 14½ days foetal age are hardly influenced by the tabby gene.

(2) The postorbital vibrissa, which is the fourth earliest in the time sequence, is almost invariably influenced by the tabby gene, whereas ulnar-carpal *a*, the sixteenth in the series, is almost invariably present.

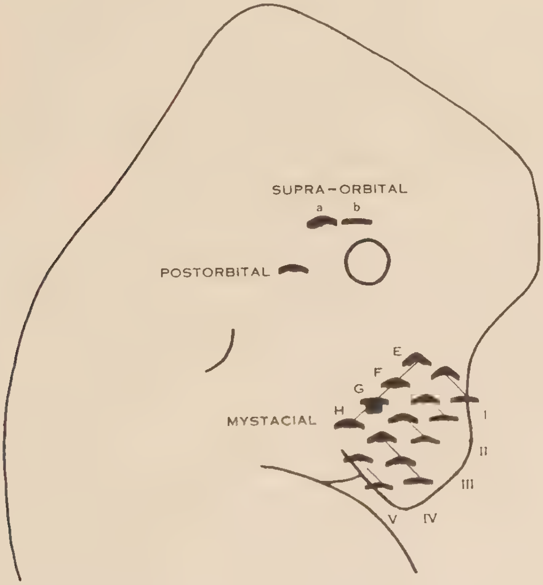


Fig. 9.—The vibrissa follicles which are present in a 12½-day normal foetus.

Thus these data give no support to the Falconer, Fraser, and King (1951) theory of action of the tabby gene (Fig. 2). The data rather indicates a localized action, a theory which is further supported by the phenotype of the tabby female. In these mice, the coat is a mosaic of normal and dark areas, the latter areas showing a fibre array similar to the whole coat of the hemizygous male (Falconer, Fraser, and King 1951). In this case normal skin and "tabby skin" are alongside each other, no doubt due to a threshold for normality being reached in some areas, not in others. Adjoining areas of skin thus demonstrate an independence in their reaction to the tabby gene, similar to that shown by the different groups of vibrissae. The correlation between heavy striping and greater loss of vibrissae in tabby females could simply mean that, in heavily striped mice, a band of tabby skin is more likely to include a vibrissal site than in mice where striping is of low intensity.

An examination of the tabby male phenotype (Fig. 5) reveals that, although there is no time-relationship between groups of vibrissae, time of initiation of follicles is important within groups. The pertinent observations are set out in Table 12. It can be readily seen that within any group of vibrissae affected by the tabby gene, the incidence of vibrissae follows the order of initiation of the follicles.

Table 12

ORDER OF FOLLICLE INITIATION WITHIN VIBRISSAL GROUPS IN THE TABBY MALE PHENOTYPE

Group	Order of Development	Percentage Incidence in Tabby Males
Supra-orbital	1. Supra-orbital *a*	100·0
	2. Supra-orbital *b*	56·8
Postoral	1. Postoral *a*	75·4
	2. Postoral *b*	0
Inter-ramal	1. Inter-ramal *b*	100·0
	2. Inter-ramal *a* and *c*	8·2
Ulnar–carpal	1. Ulnar–carpal *a*	100·0
	2. Ulnar–carpal *c*	45·9
	3. Ulnar–carpal *b*	2·7
Mystacial	1. Row I *a*	100·0
	2. Row I *b*	100·0
	3. Row I *c*	100·0
	4. Row I *d*	92·0
	1. Row II *a*	100·0
	2. Row II *b*	100·0
	3. Row II *c*	100·0
	4. Row II *d*	90·0

Another observation from Figure 5 is that there is an apparent gradient of action, from anterior to posterior, anterior mystacials being least affected while the posterior groups (postorbital and postoral) are severely depleted. Further evidence of an anterior–posterior gradient comes from the greater reduction of the growth rate of the vibrissal hairs at the postorbital and postoral sites.

The following model is therefore suggested for the action of the tabby gene and its mimic gene, crinkled. In the homozygous or hemizygous genotypes (*Ta*·, *TaTa*, *crcr*) a substance needed for both initiation and growth of follicles is produced at a slower rate than normal. This substance has a threshold level which must be present for initiation of follicles. The supply is depleted by follicle growth and, as it is produced locally in the skin, the earliest developing follicles of a group have an advantage. At 12 days, when follicles commence growth, this substance has a gradient of concentration—high anterior to low posterior.

The result is that in the period 12–17 days, certain vibrissae are the only follicles to grow. The most anterior group (mystacial) is untouched (the most posterior (postorbital) is almost invariably affected) while groups of vibrissae in intermediate locations show a variable amount of loss. In these cases, incidence within a group is closely related to the time at which each member follicle commences development.

By 17 days foetal age, sufficient of the substance has been produced over the body region so that a wave of follicle growth commences and new follicles are initiated until 20 days when the supply of the substance again falls below a critical level. It is interesting to note that the postorbital follicle, which is initially depressed, appears to make a belated effort to produce a vibrissa during this second wave of follicle growth. In the same way many of the hairs in the coat of the hemizygous tabby male may be aberrant guard hairs with a retarded time of development. This may explain Falconer's (1953) observation that the internal structure of the coat hairs of the tabby males is not typical of the awl–auchene group.

One of the most interesting features of these results is that in one case ragged is a timed gene, i.e. it blocks the development of all vibrissae which commence to grow later than 12½ days foetal age. In the other case, each group of vibrissae reacts to the presence of the tabby gene in a manner independent of time, but possibly associated with chemical or physical forces related to the position of each group.

Dun (1958) and Dun and Fraser (1959) have shown that the polygenic system, which controls the number of vibrissae in normal mice, does not affect each group of vibrissae uniformly. The evidence, from selection experiments on the number of vibrissae in individual groups, suggests that there is a separate set of genes governing the development of each group of vibrissae. This situation, although surprising, is understandable when considered in relation to the developmental study. Regional forces are such that genes affecting one group of vibrissae need not necessarily influence any other group. The data from the ragged mice suggests that groups are not completely independent and one would therefore expect some degree of overlap between genetic systems. The accumulation of further data may enable such an overlap to be demonstrated.

V. ACKNOWLEDGMENTS

Much helpful advice was obtained from Mr. A. G. Lyne, Sheep Biology Laboratory, C.S.I.R.O., Prospect, N.S.W., and in particular from Miss M. J. Heideman. Much of the histological work was done by Mr. E. C. Davis, Wool Research Laboratory, Agricultural Experiment Station, Trangie, N.S.W. This work was completed while the author was a student in the Animal Genetics Section, C.S.I.R.O., University of Sydney, and my thanks are due to Dr. A. S. Fraser and Dr. J. M. Rendel for their assistance during the project.

VI. REFERENCES

CARTER, T. C., and PHILLIPS, RITA J. S. (1954).—Ragged, a semi-dominant coat-texture mutant. *J. Hered.* **45**: 151–4.

DAVIDSON, PAMELA, and HARDY, MARGARET H. (1952).—The development of mouse vibrissae *in vivo* and *in vitro*. *J. Anat., Lond.* **86**: 342–56.

DRY, F. W. (1926).—The coat of the mouse. *J. Genet.* **16**: 287–340.

DUN, R. B. (1958).—Growth of the mouse coat. VI. Distribution and number of vibrissae in the house mouse. *Aust. J. Biol. Sci.* **11**: 95–105.

DUN, R. B., and FRASER, A. S. (1958).—Selection for an invariant character, "vibrissa-number", in the house mouse. *Nature* **181**: 1018.

DUN, R. B., and FRASER, A. S. (1959).—Selection for an invariant character, "vibrissa-number", in the house mouse. *Aust. J. Biol. Sci.* **12** (in press).

FALCONER, D. S., FRASER, A. S., and KING, J. W. B. (1951).—The genetics and development of crinkled, a new mutant in the house mouse. *J. Genet.* **50**: 324–44.

FALCONER, D. S. (1953).—Total sex linkage in the house mouse. *Z. indukt. Abstamm.-u. Vereb-Lehre* **85**: 210-19.

GRÜNEBERG, H. (1943*a*).—Congenital hydrocephalus in the mouse, a case of spurious pleiotropism. *J. Genet.* **45**: 1–21.

GRÜNEBERG, H. (1943*b*).—The development of some external features in mouse embryos. *J. Hered.* **34**: 89–92.

SLEE, J. (1957).—The morphology and development of "ragged"—a mutant affecting the skin and hair of the house mouse. I. Adult morphology. *J. Genet.* **55**: 100–21.

VARIATION OF VIBRISSA NUMBER IN THE MOUSE

By A. S. Fraser,* T. Nay,* and B. Kindred*

[*Manuscript received February* 19, 1959]

Summary

Tabby (TA) and crinkled (CR) stocks were crossed to study any interaction between the *Ta* and *cr* genes in their effects on the number of vibrissae in minor groups on the face.

No consistent trend was found and the data were re-examined to consider the effects of the background genotype of the TA and CR stocks. Evidence from the F_1 between these, from selection for high and low vibrissa number on *Ta* mice, and from crossing *Ta* mice to a stock formed from rare mice with high inter-ramal score, which occur in a normal stock, indicate the presence of partially autonomous genetic systems specific for each group of vibrissae. The effects of these are shown only in *Ta* mice. In normal mice from the same litters the vibrissa number is almost invariable. The canalization of vibrissa number is considered to explain the regression to the normal number rather than to the value at the beginning of selection which occurs when the high-selection line is crossed to unselected lines.

I. Introduction

The concepts of "developmental canalization", "genetical homeostasis", and "polygenic balance" are all concerned with the ability of a population to contain more genetic variation than is expressed phenotypically under normal conditions. Such genetic variation is exhibited phenotypically only under conditions of stress, which may be imposed by (1) long continued selection (Mather 1943); (2) environmental shocks applied during early development (Goldschmidt 1935; Waddington 1953; Landauer 1958); and (3) by the introduction of a mutant gene (Landauer 1946, 1948; Goldschmidt and Piternick 1957; Dun and Fraser 1958, 1959; and Rendel 1959).

Our own studies have been centred on the number of facial vibrissae of the mouse. The secondary groups of such vibrissae are extremely constant in number (Dun 1959) in the absence of mutant genes. The tabby (*Ta*) and crinkled (*cr*) genes both cause a reduction in the number of secondary vibrissae which is accompanied by a marked increase of variability. This variability is, at least in part, genetic (Dun and Fraser 1958, 1959).

The tabby and crinkled genes are close mimics affecting similarly many aspects of the coat and skin. These effects have been described fully by Falconer, Fraser, and King (1951). Falconer (personal communication) in a comparison of *Ta*+ . ++ and *Ta*+ . +*cr* found no evidence of interaction for the effect of these genes on the pattern of coat colour. This does not preclude the possibility of interaction occurring at increased gene dosages, with respect to other aspects of the phenotype.

---

* Animal Genetics Section, C.S.I.R.O., University of Sydney.

In this paper, a series of crosses between the tabby and crinkled stocks, which produced multiple combinations of both genes, are discussed with reference to (i) possible interactions of *Ta* and *cr*, and (ii) background modifiers.

II. Material and Methods

Two stocks of mice were used—TA and CR—which are maintained in segregation for *Ta* and *cr* respectively. These stocks are not related to each other or to specific inbred lines, and can be considered to be markedly heterogenous.

Table 1

average numbers of vibrissae in the TA and CR stocks

Stock	Genotype	Total	Position			
			A	B	C	D
TA	++ (♂, ♀)	13	4	2	4	3
	Ta+ (♀)	9·4	3·7	1·8	2·2	1·6
	Ta· (♂)	6·7	3·7	0·3	1·5	1·2
	Ta Ta (♀)	6·8	3·8	0·4	1·5	1·2
CR	++ (♂, ♀)	13	4	2	4	3
	+*cr* (♂)	13	4	2	4	3
	+*cr* (♀)	13	4	2	4	3
	crcr (♀)	6·3	2·7	0·6	1·0	1·9
	crcr (♂)	6·3	2·8	0·6	1·1	1·8

The formation of the various combinations of *Ta* and *cr* was achieved by a series of crosses and backcrosses. These were called the TC stock, and all mice in this stock were derived from TA × CR crosses and were rarely more than a few generations from the parental stock.

The occurrence of the vibrissae was scored at 8–10 days after birth. All the scoring was done by T.N., and, although a subjective element always enters into such scores, the restriction of scoring to one person reduces this danger.

Four groups of vibrissae were scored: the supra- and postorbitals, the postorals, and the inter-ramals. To simplify terminology these are termed the A, B, C, and D groups respectively. The first three groups occur on both sides of the face; the D group occurs at a single position under the chin. There are two vibrissae at each of the A and C positions, one at each of the B positions, and three at the D position, in normal mice (see Dun 1959).

In several sets of data from other experiments, included to expand and corroborate conclusions derived from the basic set of data, vibrissae were scored by a group of operators who continually checked their decisions against each other. In these data a fifth group (E) of vibrissae was scored—the ulnar–carpals, located on the fore feet.

III. Results and Discussion

(*a*) *Effects of* Ta *and* cr *Genes*

The average total number of secondary vibrissae in TA and CR stocks and the average number for each of the positions A, B, C, and D are given in Table 1. These data show that:

- (i) No sex difference occurs in expression (compare *Ta·* with *TaTa* and *crcr* (♂) with *crcr* (♀)).
- (ii) The *Ta*+ genotype is roughly intermediate between *TaTa* and ++ for total vibrissa number, whereas the *cr* gene is a complete recessive.
- (iii) The absence of dominance of the *Ta* gene for total vibrissa number does not apply to the separate groups of vibrissae. The gene is completely dominant, but of low penetrance, for the A vibrissae, partially recessive for the B vibrissae, and partially dominant for the C and D vibrissae.

Table 2

FREQUENCY DISTRIBUTIONS OF TOTAL NUMBER OF VIBRISSAE

Genotype	Total Number of Vibrissae:											
	2	3	4	5	6	7	8	9	10	11	12	13
TaTa . ++	—	—	—	7	10	29	6	3	—	—	—	—
Ta· . ++	—	—	—	13	17	29	7	4	2	—	—	—
Total	—	—	—	20	27	58	13	7	2	—	—	—
++ . *crcr* (♀)	—	1	4	3	6	4	3	2	1	—	—	—
+· . *crcr* (♂)	—	4	5	4	4	3	7	5	1	—	—	—
Total	—	5	9	7	10	7	10	7	2	—	—	—

Although the *Ta* and *cr* genes are similar in homozygous expression for total number of vibrissae—6·75 compared with 6·3—this similarity does not hold for the individual vibrissa groups. The *cr* gene differs markedly from the *Ta* gene in its much greater effect on the A vibrissae. There are consistent but smaller differences between the genes in their effects on the B, C, and D vibrissae.

A marked difference between the *Ta* and *cr* genes occurs in variability of vibrissa number. This is shown in Table 2. Although there is little difference in the mean number of vibrissae between the two types, the range is greater in *crcr* mice, and there is no marked mode as occurs in *Ta* mice.

(*b*) *Interaction of* Ta *and* cr *Genes*

The average total number of secondary vibrissae in TA and CR stocks and the average number for each of the four positions are given in Table 3. The total number of vibrissae shows no evidence of any easily understood interaction;

increased substitution of either *cr* or *Ta* may increase or decrease the total number of vibrissae. Examination of the means of the four types of vibrissae does not indicate any resolution of this complexity.

So far these data have been discussed solely in terms of all differences being due to the effects of the *Ta* and *cr* genes. Dun and Fraser (1958, 1959) have shown, however, that the degree of effect of the *Ta* gene can be considerably modified by selection, i.e. there is a genetic system, which is not manifested in normal mice, being exhibited only in the presence of the *Ta* gene. Presumably the same or similar systems are manifested in crinkled mice. It is therefore plausible to re-examine these data from this viewpoint, namely, that differences in expression between tabby and crinkled mice are determined, not by differences between *Ta* and *cr*, but by differences in the constitution of a background genotype.

Table 3

AVERAGE NUMBERS OF VIBRISSAE IN VARIOUS GENOTYPES ARRANGED TO SHOW ANY EFFECT OF INCREASING DOSAGE OF *cr* ON THE EXPRESSION OF *Ta*, AND OF *Ta* ON THE EXPRESSION OF *cr*

Genotype	Total	Position			
		A	B	C	D
Ta+ . ++ (♀)	9·4	3·7	1·8	2·2	1·6
Ta+ . +*cr* (♀)	10·9	3·9	1·9	2·8	2·2
Ta+ . *crcr* (♀)	5·6	2·8	0·1	1·0	1·7
Ta· . ++ (♂)	6·6	3·7	0·3	1·5	1·2
Ta· . +*cr* (♂)	6·1	2·9	0·3	1·4	1·4
Ta· . *crcr* (♂)	6·3	3·3	0·4	0·9	1·8
TaTa . ++ (♀)	6·8	3·8	0·4	1·5	1·2
TaTa . *crcr* (♀)	6·7	3·0	0·8	0·9	1·8
++ . *crcr* (♂, ♀)	6·3	2·75	0·6	1·05	1·85
Ta+ . *crcr* (♀)	5·6	2·8	0·1	1·0	1·7
Ta· . *crcr* (♂)	6·3	3·3	0·4	0·9	1·8
TaTa . *crcr* (♀)	6·7	3·0	0·8	0·9	1·8

(c) *Differences of Background Genotype between the TA and CR Stocks*

Comparisons between the TA and CR stocks can be made at two levels of expression. The *Ta*+ level in which the mean number of vibrissae is 9–10, and the homozygote level in which the mean number of vibrissae is 6–7. The two parental stocks, TA and CR, give estimates of the degree of effect of the background genotype. The TA stock allows estimates at both the *Ta*+ and the homozygote level. The CR stock allows estimates only at the homozygote level. The TC stock consists of F_1 and various complex matings between TA and CR. These can all be combined to give an estimate of the effect of crossing TA and CR at the *Ta*+ and homozygous levels. In this latter group, progeny from three crosses have been included which were not included above. These are mice, which though *crcr* may be either (i) *TaTa* or *Ta*+, (ii) *Ta*· or +·, and (iii) *Ta*+ or ++. Since all these mice are *crcr* they are at the homozygous level, and can therefore be included in the TC group of that level. The results are given in Table 4.

At the homozygote level the crossbred mice (TC) have, for each group of vibrissae, an intermediate expression, suggesting that the differences of expression between TA and CR are not aspects of the *Ta* and *cr* genes, but rather of the background genotype.

TABLE 4

FREQUENCY DISTRIBUTIONS OF THE NUMBER OF VIBRISSAE, GIVEN SEPARATELY FOR THE FOUR POSITIONS, COMPARED BETWEEN THE TA AND TC STOCKS AT THE *Ta*+ LEVEL, AND BETWEEN TA, TC, AND CR STOCKS AT THE HOMOZYGOTE LEVEL

Stock	Position A					Position B			Position C					Position D			
	4	3	2	1	0	2	1	0	4	3	2	1	0	3	2	1	0
TA	83	8	8	2	0	89	3	8	9	8	77	3	2	11	41	48	—
TC	93	3	3	—	—	95	1	3	30	19	51	—	—	30	65	6	—
TA	84	8	7	1	—	11	12	78	—	—	62	20	17	—	17	83	—
TC	43	22	31	1	0·5	12	15	72	—	—	47	19	33	2	61	36	0·5
CR	31	12	57	—	—	21	19	59	—	—	45	18	37	6	72	22	—

This is supported if we separate the F_1 matings (TA × CR) from the other matings of the TC stock which comprise various backcrosses and intercrosses, none of which from the F_1 matings produced sufficient progeny to justify consideration as a separate group. The comparisons of TA, CR, and TC (F_1) are shown in Table 5. There were 21 F_1 matings, comprising 34 per cent. of all TC matings, and it can be seen from comparison of Tables 4 and 5, that the F_1 results, on the average, when compared to the overall TC group, regress towards the TA degree of expression, which is expected, since the majority of the TC matings include one or more backcrosses to the CR stock.

(*d*) *Specificity of Genetic Systems Affecting Vibrissa Number*

These data indicate the existence of polygenic differences between the TA and CR stocks, which, though having no effects on the number of vibrissae in normal mice, markedly affect the expression of the *Ta* and *cr* genes. Dun and Fraser (1958, 1959) have shown in a selection experiment based on the total number of secondary vibrissae, that such polygenic systems exist, and that they can affect the number of vibrissae in the absence of the *Ta* gene, if sufficiently modified by selection. Separate selection experiments, based initially on rare mice from normal stocks having varied vibrissae numbers, were initiated for two groups of vibrissae—the B and D groups respectively. In both experiments selection has produced differences of vibrissa number. In the D selection, where the normal number is three, mice with four such vibrissae are frequent in the high line, and mice with two vibrissae are frequent in the low line. No changes have been noted in the number of any other vibrissae, apart from two mice produced in the last, the seventh, generation of selection. One of these lacked a single ulnar–carpal vibrissa, the other lacked a single supra-orbital vibrissa. Considering the extent of the differences produced, it is clear that the number of D vibrissae has a considerable autonomy of genetic control. In the B selection, where the normal number is four, mice with six such vibrissae are frequent in the high line, and mice with less than four B vibrissae are frequent in the low line. In this stock there have always been mice with different-than-normal numbers of vibrissae in the D group, indicating that the polygenic system operated on here has a wider effectiveness than the one under selection in the D line.

TABLE 5

FREQUENCY DISTRIBUTIONS OF THE NUMBER OF VIBRISSAE, GIVEN SEPARATELY FOR EACH POSITION, COMPARED BETWEEN TA AND CR STOCKS AND BETWEEN TA × CR (F_1) AT THE HOMOZYGOTE LEVEL

Stock	Position A					Position B			Position C					Position D			
	4	3	2	1	0	2	1	0	4	3	2	1	0	3	2	1	0
TA	84	8	7	1	—	11	12	78	—	—	62	20	17	—	17	83	—
TA × CR (F_1)	44	21	34	—	—	15	3	81	—	—	52	27	20	—	38	57	3
CR	31	12	57	—	—	21	19	59	—	—	45	18	37	—	72	22	—

Crosses have been made between the low line (LST) of selection for total vibrissa number, and the high (HD) and low (LD) lines of selection for the number of D vibrissae. The frequencies of mice with different vibrissa numbers in the LST and F_1 progeny are given in Table 6. In all five groups of vibrissae there is a difference between the LST × HD, and LST × LD crosses in the direction that one would expect, namely that the former mice have less vibrissae missing than the latter. The differences are small in the A and B groups, and could be fortuitous sampling errors. The differences in the C, D, and E groups are large, showing that the HD and LD lines differ in the genetic systems controlling these three groups. These differences are not apparent in +/+ mice (HD and LD differ only in the numbers of D vibrissae), such differences becoming phenotypically expressed only at the *Ta*+ level. We can conclude that although there is a considerable autonomy

TABLE 6

FREQUENCIES OF $Ta+$ MICE HAVING DIFFERENT NUMBERS OF VIBRISSAE IN THE LOW-SELECTION LINE (LST) FOR TOTAL NUMBER OF VIBRISSAE, AT GENERATION FIVE, AND IN THE F_1 MICE PRODUCED BY CROSSING AT THIS GENERATION TO THE HIGH (HD) AND LOW (LD) LINES OF SELECTION FOR INTER-RAMAL VIBRISSAE NUMBER (D)

Stock	Position A					Position B			Position C					Position D				Position E							n
	4	3	2	1	0	2	1	0	4	3	2	1	0	3	2	1	0	6	5	4	3	2	1	0	
LST × HD	100	—	—	—	—	100	—	—	46	15	35	4	—	54	30	15	—	76	17	7	—	—	—	—	46
LST × LD	88	7	4	—	—	94	6	—	13	14	72	—	—	4	30	65	—	38	25	36	1	—	—	—	69
LST	79	16	5	—	—	92	5	3	—	16	82	3	—	5	26	68	—	11	26	58	5	—	—	—	38

TABLE 7

FREQUENCIES OF MICE HAVING DIFFERENT NUMBERS OF VIBRISSAE IN THE HIGH- (HST) AND LOW- (LST) SELECTION LINES FOR TOTAL VIBRISSA NUMBER AT GENERATION FIVE OF SELECTION, AND IN THE F_1 MICE PRODUCED BY CROSSING AT THIS GENERATION TO THE A, C_{57}, DBA, AND Aw101 INBRED LINES

Stock	Position A					Position B			Position C					Position D				Position E							n
	4	3	2	1	0	2	1	0	4	3	2	1	0	3	2	1	0	6	5	4	3	2	1	0	
HST	98	2	—	—	—	91	2	7	11	16	73	—	—	41	38	21	—	73	11	16	—	—	—	—	44
HST × inbred lines	98	2	—	—	—	92	8	—	33	17	49	—	—	30	46	24	—	81	10	8	2	—	—	—	63
LST × inbred lines	89	9	2	—	—	93	2	5	25	18	57	—	—	25	34	41	—	56	25	16	5	—	—	—	44
LST	79	16	5	—	—	92	5	3	—	16	82	3	—	5	26	68	—	11	26	58	5	—	—	—	38

in the genetic control of the number of D vibrissae, this is not complete, i.e. selection for differences in the number of D vibrissae does produce changes in the genetic system controlling the C and E vibrissae, which are only expressed in *Ta*+ mice, at that degree of selection. As selection is continued it is probable that such differences will become phenotypically expressed in +/+ mice.

We can conclude from these data that the polygenic basis of secondary vibrissa number is separated into a number of distinct systems which are to varying extents autonomous for specific vibrissa groups.

(*e*) *Regression towards the Norm*

An analogy can be drawn between the regression towards the norm found in these data (Table 4), and that which occurs in selected lines after relaxation of selection pressure (see Lerner 1954). The occurrence of such a regression may be differently determined in the two materials, but it is possible that the regression which occurs in "relaxed" selection lines is similarly due to a mechanism of canalization whose action is not as intense as that determining the number of secondary vibrissae. This possibility can be investigated in selection experiments utilizing a phenotype shift introduced by a mutant gene (Dun and Fraser 1958, 1959; Rendel 1959). In these experiments the character under selection is strongly canalized as shown by the absence of phenotypic variation in the presence of genetic variation. Introduction of a mutant gene causes a shift of the phenotype with a concomitant marked increase of phenotypic variability. Selection is then applied from a starting point which is outside the zone of canalization, and being directed at both an increase and a decrease of the character, it will shift the character towards, and away from, the zone of canalization. Consequently, if selection is relaxed, then the character may (i) shift towards the starting point of selection, or (ii) shift towards the normal phenotype. The former will show that the regression which occurs on relaxation of selection has a different basis to that which occurs because of developmental canalization. The latter will show that the two regressions both have a basis in the canalization mechanism. Rendel (1959, and personal communication) maintained his "scute" selection experiment in several lines which were, at times, crossed. Such crossing, which is a relaxation of selection, always resulted in a regression towards the starting point of selection, not towards the zone of canalization.

We have several experiments in progress involving crosses between our vibrissa-selection lines (see Dun and Fraser 1959). The first of these involved taking *Ta*· mice from the high- and low-selection lines and crossing these to several inbred lines. Dun (1959) has shown that the secondary groups of vibrissae in inbred lines do not differ in any but minor aspects from crossbred mice. Consequently we can assume that the inbred populations are a random sample of genotypes around the mean value of crossbred populations. Crossing the two selected lines to these inbred lines should, therefore, result in a regression towards the starting point of selection.

The results are given for five groups of secondary vibrissae in Table 7. These results show, in crosses involving the selection line for increased number of vibrissae, a decided regression towards the normal vibrissa number (towards the zone of canalization), not towards the starting point of selection. There is, in the crosses involving the selection line for increased number of vibrissae, an increased rather then a decreased, number of vibrissae. In crosses involving the selection line for decreased number of vibrissae, the crosses also show an increased number of vibrissae; this may be a regression towards the norm, or towards the starting point of selection.

Rendel's and our own results show that two mechanisms of regression can be operative; one of canalization at the normal value of the character, the other an inherent feature of the genetic system determining the phenotype, which is independent of the phenotypic level at which the genotype is operative. It is difficult to conceive that the latter is due to selection pressures acting directly on the genetic system, since this would involve the mutant level of expression having a selective advantage over the normal level. It is equally difficult to consider a selective advantage of heterozygosity *per se* as the explanation, since this would involve heterozygotes being advantageous at two different levels of expression. It is more plausible to suggest that such regression is due to selection operating on linked "fitness" genes whose equilibria have been disrupted by the directed selection.

The regression, in our own data, away from the starting point of selection, towards the normal number of vibrissae shows (1) that the mechanisms of canalization are operative at the normal level in the presence of the mutant gene, i.e. the mutant gene does not act on the mechanism of canalization as is suggested by Dun and Fraser (1959); and (2) that the effectiveness of the mechanisms of canalization extends considerably beyond the normal range of vibrissa number.

IV. References

Dun, R. B. (1959).—The development and growth of vibrissae in the house mouse with particular reference to the time of action of the tabby (*Ta*) and ragged (*Ra*) genes. *Aust. J. Biol. Sci.* **12**: 312–30.

Dun, R. B., and Fraser, A. S. (1958).—Selection for an invariant character, "vibrissa-number", in the house mouse. *Nature* **181**: 1018–19.

Dun, R. B., and Fraser, A. S. (1959).—Selection for an invariant character, "vibrissa-number", in the house mouse. *Aust. J. Biol. Sci.* **12** (in press).

Falconer, D. S., Fraser, A. S., and King, J. W. B. (1951).—The genetics and development of crinkled, a new mutant in the house mouse. *J. Genet.* **50**: 324–44.

Goldschmidt, R. (1935).—Gen und Ausseneigenschaft (Untersuchungen an *Drosophila*). *Z. indukt. Abstamm.-u. VererbLehre* **69**: 38–131.

Goldschmidt, R., and Piternick, L. K. (1957).—The genetic background of chemically induced phenocopies in *Drosophila*. *J. Exp. Zool.* **135**: 127–202.

Landauer, W. (1946).—The results of selection against the expression of the "short upper beak" mutation of fowl. *Amer. Nat.* **80**: 490–4.

Landauer, W. (1948).—The phenotypic modification of hereditary polydactylism of fowl by selection and by insulin. *Genetics* **33**: 133–57.

Landauer, W. (1958).—On phenocopies, their development, physiology and genetic meaning. *Amer. Nat.* **92**: 201–13.

Lerner, I. M. (1954).—"Genetic Homeostasis." (Oliver & Boyd: Edinburgh.)

Mather, K. (1943).—Polygenic inheritance and natural selection. *Biol. Rev.* **18**: 32–64.

Rendel, J. M. (1959).—The canalization of the scute phenotype. *Evolution* (in press).

Waddington, C. H. (1953).—Genetic assimilation of an acquired character. *Evolution* **7**: 118–26.

SHORT COMMUNICATIONS

NEUTRAL AND ACIDIC AUXINS IN DEVELOPING TOBACCO FRUIT*

By L. G. Paleg† and R. M. Muir‡

Indolylacetic acid (IAA) has been postulated as the enzymatically produced endogenous auxin controlling the development of fruit (Muir 1947). Few reports have appeared suggesting that there may, in fact, be other auxins associated with this function particularly during its earliest phase, between pollination and fertilization.

Recently, Lund (1956*a*, 1956*b*), in an investigation of the hormonal complement of young Maryland Mammoth tobacco fruit, reported that IAA was the only auxin responsible for the control of processes associated with fruit development, its concentration beginning to rise about 50 hr after pollination, coincident with the beginning of fertilization and the start of ovary enlargement. However, a compound similar to indolylacetonitrile (IAN) was also found in the acid fraction of ether extracts (although its concentration did not vary) while the neutral extracts contained no activity as measured by the *Avena* curvature test. Even though IAN is a neutral compound some of it may enter the acidic fraction (Bennet-Clark and Kefford 1953; Cartwright, Sykes, and Wain 1956). However, if it is present at all, it is unlikely that none of it would be found in the neutral ether extract.

The detection and quantitative estimation of neutral auxins with the curvature test is complicated by the tendency of the neutral compounds towards non-polar or lateral movement (Bentley and Bickle 1952). For this reason the straight growth of *Avena* coleoptiles was adopted in this work as a more suitable means of determining the changes occurring in the acidic and neutral auxin fractions of Little Turkish tobacco ovary tissue during the first 4 days following pollination.

Ovary tissue was lyophilized and ground to pass a 40-mesh screen prior to extraction with dry, peroxide-free ether for 12 hr at −20°C. The ether was partitioned three times with 1 per cent. sodium bicarbonate to remove the acid fraction and the sodium bicarbonate was then acidified and extracted three times with fresh ether. All ether solutions were reduced in volume to 5 ml over a hot water-bath at which time 10 ml distilled water was added. The remaining ether was evaporated, and, following cooling, the water solutions were adjusted to pH 6·0. As a routine procedure, 1 : 10 and frequently 1 : 100 dilutions of the water solutions were assayed with the *Avena* straight-growth test (Muir and Hansch 1953), to be certain that the extracts did not contain supra-optimal concentrations of hormones.

---

* Manuscript received April 13, 1959.

† Department of Botany, State University of Iowa, U.S.A.; present address: Waite Agricultural Research Institute, University of Adelaide.

‡ Department of Botany, State University of Iowa, U.S.A.

Through analysis of unpollinated ovary tissue at 1, 2, 3, and 4 days after anthesis, it was determined that the free auxin levels (both acidic and neutral) in 200 mg dry weight (about 30 ovaries) were below the limits of sensitivity of the test. However, as shown in Figure 1(*a*), there is, immediately following pollination (carried out at anthesis), a rapid production of neutral auxin. This exceeds acid auxin production and by the third day following pollination, neutral auxin level is twice that of acid auxin. Four days after pollination, coincident with increasingly rapid changes in dry weight (Fig. 1(*b*)), a diminution in neutral auxin and a very rapid rise in acid auxin content occur. This is evident on both a per unit tissue and a per ovary basis.

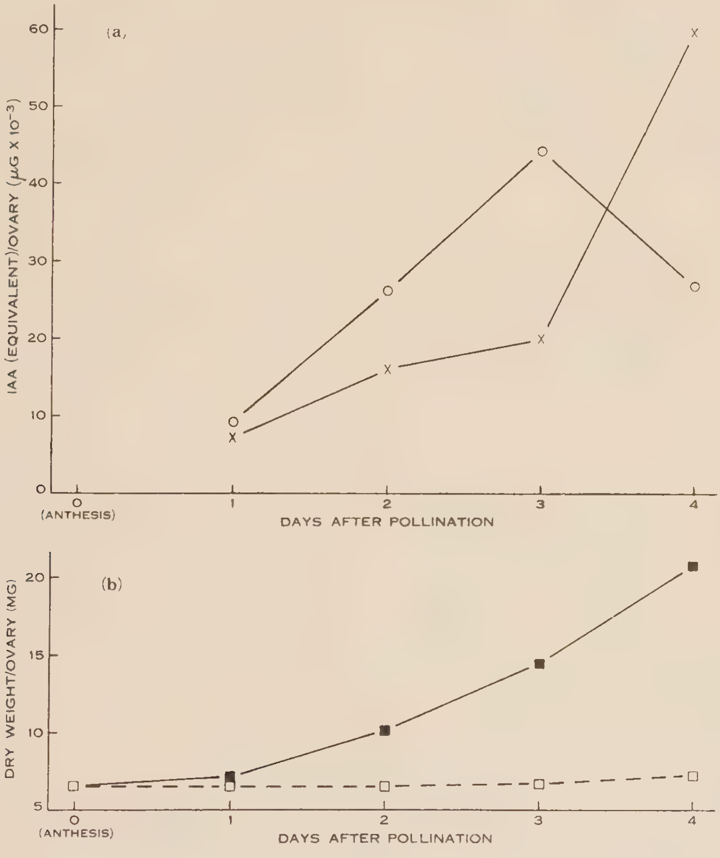


Fig. 1.—(*a*) Changes in acid (×) and neutral (○) auxin levels following pollination, calculated as $\mu g \times 10^{-3}$ IAA per ovary. (*b*) Changes in dry weight of unpollinated (□) and pollinated (■) ovaries.

Three interpretations of the results are possible:

(1) Neutral auxin functions as a precursor in the formation of acid auxin, conversion being initiated or heightened in conjunction with embryo and endosperm development (beginning in the majority of ovules between the second and third days following pollination).

(2) The biosynthetic pathways lead from a precursor common to both acid and neutral auxin, and the amounts of each present serve as an indication of the success of the competing systems.

(3) The syntheses of acid and neutral auxins are unrelated in so far as different phenomena may control their initiation. The formation of the neutral fraction may be predominantly a pre-fertilization process, while the formation of acid auxin may be largely correlated with post-fertilization processes.

Unfortunately, the data at this time do not allow a distinct choice to be made. There are, however, similarities in all three interpretations; production of neutral auxin in the ovary is initiated by pollen germination and tube growth, and formation of acid auxin in the ovary is stimulated by fertilization and accompanying processes.

Table 1

TRYPTOPHAN CONTENT OF TOBACCO OVARIES FOLLOWING ANTHESIS

Samples for two successive days are combined

Days after Anthesis	Condition	Tryptophan (μg per ovary)		
		Total	Free	Protein*
3 and 4	Unpollinated	5·1	4·5	0·6
5 and 6	Unpollinated	13·0	10·5	2·5
1 and 2	Pollinated	23·0	3·2	19·8
5 and 6	Pollinated	252·0	25·0	227·0

* Derived by subtracting free from total tryptophan values.

The absence of auxin activity in the neutral fraction, as described by Lund (1956*a*), may have been the result of a failure of the *Avena* curvature test to assay auxins which are not transported in a strictly polar manner. It may also have been due to a supra-optimal concentration of neutral auxin (Bentley and Bickle (1952) have reported that curvature of *Avena* coleoptiles may disappear completely when IAN is supplied in supra-optimal amounts) since the neutral extracts were neither diluted before assay nor subjected to chromatographic analysis (Lund, personal communication). The slight but constant activity ascribed to IAN in the acid fraction examined by Lund might represent the partition coefficient of IAN between the acid and neutral extracts.

The time at which acidic auxin concentration in the ovary increases agrees well with Lund's results, since he also reports very little change until the pollen tubes enter the ovary and fertilization takes place. The results indicate that the initial phases of ovary enlargement (and possibly the prevention of abscission) may be under the control of neutral auxin, and that the role of acid auxin in the ovary (probably IAA) may not become active until fertilization occurs.

Since tryptophan might serve as a precursor for both neutral and acidic auxins, the amounts of tryptophan in unpollinated and pollinated ovaries were determined (Stokes *et al.* 1945; Difco Laboratories 1948; Nitsch 1951) at successive time intervals. Unfortunately, no precursor function can be ascribed to tryptophan due to the disparity, in absolute amounts, between it and the auxins. However, the results in Table 1 show that although there was a slight increase in tryptophan content in the unpollinated ovary tissue following anthesis, it was in the free or unbound form. In the interval between pollination and fertilization Lund (1956*b*) reports only a slight increase in total tryptophan content, most of it in the free form, whereas the increase in tryptophan following fertilization was entirely in the protein form. However, in the present experiments there was a greater increase in the protein tryptophan than in the free tryptophan, both before and after fertilization, indicating that in addition to effects on auxin production, the pollen is effective in stimulating amino acid metabolism and protein synthesis.

References

BENNET-CLARK, T. A., and KEFFORD, N. P. (1953).—*Nature* **171**: 645.

BENTLEY, J., and BICKLE, A. (1952).—*J. Exp. Bot.* **3**: 424.

CARTWRIGHT, P., SYKES, J. T., and WAIN, R. L. (1956).—In "Chemistry and Mode of Action of Plant Growth Substances." (Ed. R. L. Wain and F. Wightman.) (Butterworths Scientific Publications: London.)

DIFCO LABORATORIES (1948).—"Media for the Assay of Vitamins and Amino Acids." Pamph. No. 128. (Difco Laboratories: Detroit, Mich.)

LUND, H. (1956*a*).—*Amer. J. Bot.* **43**: 562.

LUND, H. (1956*b*).—*Plant Physiol.* **31**: 334.

MUIR, R. M. (1947).—*Proc. Nat. Acad. Sci., Wash.* **33**: 303.

MUIR, R. M., and HANSCH, C. (1953).—*Plant Physiol.* **28**: 218.

NITSCH, J. P. (1951).—*C.R. Soc. Biol., Paris* **145**: 1809.

STOKES, J., GUNESS, M., DWYER, I., and CASWELL, M. (1945).—*J. Biol. Chem.* **160**: 35.

DIFFERENTIAL SPECIFICITY EXHIBITED BY TWO GERMINATION INHIBITORS PRESENT IN *ECHIUM PLANTAGINEUM* L.*

By L. A. T. BALLARD† and A. E. GRANT LIPP†

The widespread occurrence of seed-germination inhibitors which come from sources external to seeds, and which apparently control germination by inhibiting embryo growth (mainly the radicle) once it has started (Moewus and Schader 1951; Mayer and Evenari 1952) is well appreciated. Examples of this class of inhibitor have been detected both in the juices or extracts of fruits or other structures derived from parts of flowers (Evenari 1949; Moewus, Moewus, and Schader 1951), and in vegetative structures (Konis 1947; Winter and Sievers 1952; Yardeni and Evenari

---

* Manuscript received April 13, 1959.

† Division of Plant Industry, C.S.I.R.O., Canberra.

1952; Bublitz 1953; Winter and Bublitz 1953). It is also recognized that they are largely non-specific, in that a given inhibitor may react differently against different seeds, as well as a given seed showing different sensitivity to different inhibitors (Evenari 1949; Koller 1955; Toole *et al.* 1956). It does not appear to have been explicitly stated that both these aspects of non-specificity may be observed in a single source. Evidence to this effect is presented in this communication.

TABLE 1

INHIBITION OF SEED GERMINATION BY WATER AND ALCOHOL FRACTIONS OF E. PLANTAGINEUM EXTRACT

Concentration of both fractions equivalent to 0·1 g dry weight of *E. plantagineum* per ml

Species	Inhibition* by:	
	Water Fraction	Alcohol Fraction
Echium plantagineum L.	++	±
Cucumis sativus L.	+++	±
Cynoglossum australe R.Br. var. *drummondii* (Benth.) Brand	+++	±
Triticum aestivum L. 'Koala'	+++	±
Brassica sp. 'Mustard Greens'	+++	+
Avena sativa L. 'Victory'	++	±
Lycopersicon esculentum Mill. 'Marglobe'	++	±
Lolium rigidum Gaud.	+	±
Trifolium subterraneum L. 'Mount Barker'	+	±
Lepidium hyssopifolium Desv.	±	±
Medicago tribuloides Desr.	±	±
Allium cepa L. 'White Pearl'	±	±
Daucus carota L. 'Chantenay'	+	+
Marrubium vulgare L.	+++	++
Brassica campestris L. 'Purple Globe'	+++	++
Phalaris tuberosa L.	++	++
Salvia verbenaca L.	+++	+++
Lactuca sativa L. 'Imperial 847'	+++	+++
Trifolium glomeratum L.	++	+++
Lepidium sativum L.	±	+++

* Germination of treated seeds as per cent. of control germination. +++, <25 per cent.; ++, 25–50 per cent.; +, 50–75 per cent.; ±, >75 per cent.

Experimental

Air-dried, ground aerial parts of *Echium plantagineum* were extracted overnight at room temperature with 80 per cent. ethanol and filtered. The extraction was repeated twice and the filtrates combined. The alcohol was removed under reduced pressure at a temperature not greater than 50°C, and the residue taken up in water. This *crude extract* was fractioned by passage down a column of activated carbon and "Hyflo Supercel", and the eluate and water washings combined to give a *water fraction*. The extruded column was boiled for approximately 1 min in 80 per cent.

ethanol, filtered, the alcohol removed under reduced pressure, and the residue taken up in water to give an *alcohol fraction*. Extracts or fractions to be chromatographed were applied as streaks to Whatman No. 1 filter paper and chromatograms developed in ethanol–water (80 : 20 v/v). The activities of extracts, fractions, and chromatograms were assessed by the germination responses of the seeds of species listed in Table 1. These were set out on filter paper moistened with distilled water, extract, or fraction, or on strips cut from chromatograms moistened with distilled water. The seeds of *Trifolium subterraneum* L. and *Medicago tribuloides* Desr. were incubated in the dark at 20°C, and the remainder at 25°C. Those of *Salvia verbenaca* L., after 2 days in the dark at 25°C, were transferred to artificial light at 20°C (8 hr per day at approximately 1500 f.c.).

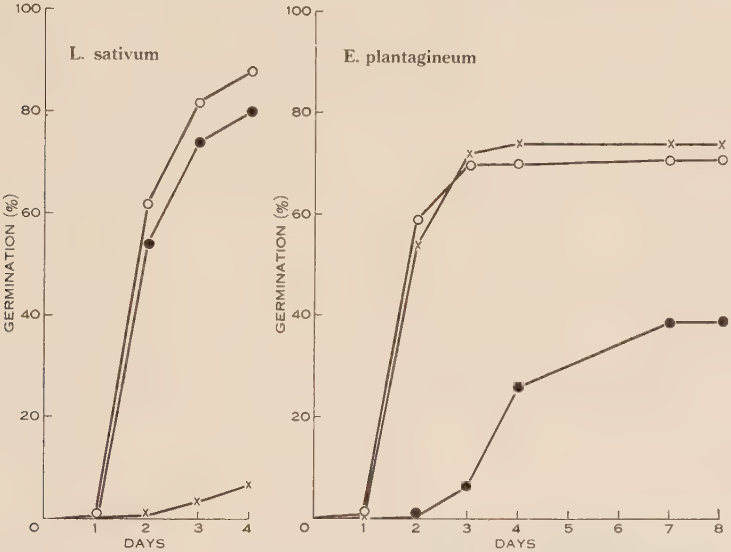


Fig. 1.—Germination of *E. plantagineum* and *L. sativum* seeds in distilled water (○), water fraction (●), and alcohol fraction (×), both fractions at a concentration equivalent to 0·1 g dry weight of *E. plantagineum* per ml.

Evidence for Two Inhibitors

From typical results presented in Figure 1 it is seen that the water fraction reduces germination of *E. plantagineum* to about half that of the water control, but has no significant effect on *Lepidium sativum* L. germination. The alcohol fraction behaves conversely.

On chromatograms of crude extract, an inhibitor of *E. plantagineum* is found at R_F 0·3, and of *L. sativum* at R_F 0·7–0·8 (Fig. 2). When chromatograms from both fractions are assayed separately, *E. plantagineum* is inhibited at R_F 0·3–0·4 in the water-fraction chromatograms and nowhere in those of the alcohol fraction; *L. sativum* is inhibited at R_F 0·7–0·8 in the alcohol-fraction chromatograms and nowhere in those of the water fraction.

Species Response

Differential sensitivity to the two inhibitors is indicated by the results of Table 1. Germination responses depend on external inhibitor concentration; at the concentration employed most species are like *E. plantagineum* in being more sensitive to the water-fraction inhibitor, and only *Trifolium glomeratum* approaches *L. sativum* in being more sensitive to the alcohol-fraction inhibitor. Others are either relatively insensitive, or equally sensitive, to both inhibitors.

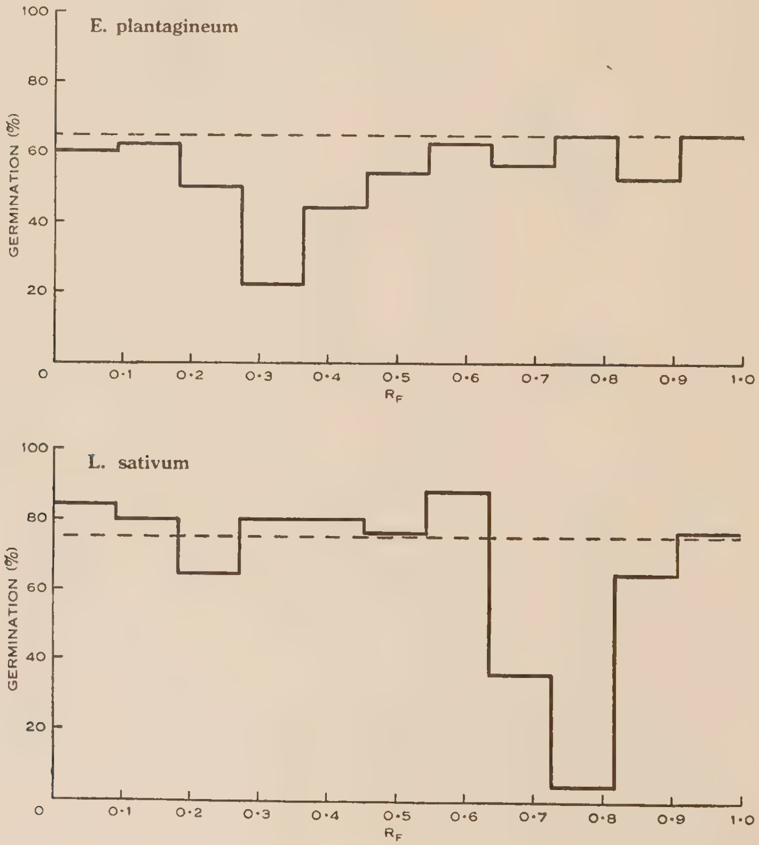


Fig. 2.—Germination on day 3 of test of *E. plantagineum* and *L. sativum* seeds on strips cut from chromatograms of crude extract. Broken lines represent mean germination on paper through which pure solvent had passed.

The inhibitors present in aerial parts of *E. plantagineum* are distinguishable chemically by their behaviour on carbon columns and on paper chromatograms, and biologically by the differential responses to them of seeds of a number of species. Both inhibitors are water soluble, stable to alcohol, and to temperatures of at least 50°C, and to prolonged storage at −10°C. They have not been further characterized.

References

BUBLITZ, W. (1953).—*Naturwissenschaften* **40**: 275–6.

EVENARI, M. (1949).—*Bot. Rev.* **15**: 153–94.

KOLLER, D. (1955).—*Bull. Res. Coun. Israel* **4**: 379–87.

KONIS, E. (1947).—*Palest. J. Bot.* J **4**: 77–85.

MAYER, A. M., and EVENARI, M. (1952).—*J. Exp. Bot.* **3**: 246–52.

MOEWUS, F., MOEWUS, L., and SCHADER, E. (1951).—*Z. Naturforsch.* **6B**: 261–70.

MOEWUS, F., and SCHADER, E. (1951).—*Ber. dtsch. bot. Ges.* **64**: 124–9.

TOOLE, E. H., HENDRICKS, S. B., BORTHWICK, H. A., and TOOLE, V. K. (1956).—*Annu. Rev. Pl. Physiol.* **7**: 299–324.

WINTER, A. G., and BUBLITZ, W. (1953).—*Naturwissenschaften* **40**: 416.

WINTER, A. G., and SIEVERS, E. (1952).—*Naturwissenschaften* **39**: 191–2.

YARDENI, D., and EVENARI, M. (1952).—*Phyton* **2**: 11–16.

TRANSLOCATION OF LABELLED ASSIMILATES IN THE SOYBEAN

By R. Thaine,* Stella L. Ovenden,† and J. S. Turner†

[*Manuscript received July* 3, 1959]

Summary

When $^{14}CO_2$ was supplied to a limited area of a mature illuminated leaf of soybean, the radioactive compounds appeared rapidly in the root, the apex of the shoot, and in young unexpanded leaves. Translocation to these "sinks" took place in the light, during photosynthesis at the source.

Translocation also went on in darkness subsequent to fixation in the leaf, and after 24 hr the concentration of mobile tagged compounds in the source leaf was very low. Considerable activity, however, remained at the source in the form of insoluble compounds which were not starch. It is concluded that masking lowers the concentration of mobile assimilates in leaves.

In the soybean the vascular pattern is such that movement of assimilate from the source leaf to any other leaf can occur. Movement into mature leaves was very small in extent when compared to movement into growing points or expanding leaves. Nevertheless, movement into primary and trifoliate mature leaves, or from one leaflet to another of a mature leaf, can be demonstrated if $^{14}CO_2$ of sufficiently high activity is applied to the source leaflet, and especially if other sinks for assimilate are removed.

Movement of tagged assimilate into a mature leaflet was clearly demonstrated, even when applied at low activity, when the leaf showed necrotic spots (presumably due to nutrient imbalance). Radioactivity then appeared at a high level around the necrotic areas.

Movement into the apical halves of source leaves is much smaller in extent than is basipetal movement. Acropetal movement may, however, be demonstrated and its extent is increased by shading. In general, the shading of a mature leaf increases the tendency of tagged assimilate to move into it, presumably because shading lowers the total concentration of assimilates.

The *removal* of all mature leaves between source and apex significantly increased movement to the apex. As the leaves removed normally import little, it is presumed that this result is due to the lowering of the concentration of total assimilate at the apex resulting from the removal of leaves which would normally export untagged assimilate.

Shading of the green apex, in many but not in all experiments, led to a *decreased* movement to the apex from the mature source leaf. This anomalous result was not established as statistically significant and needs further investigation.

The direction of movement from a given leaf depends upon the age and position of the leaf on the stem. A young leaf forms part of the major apical sink for upward-moving assimilate and for its own photosynthetic products, exporting nothing to the rest of the plant. When it has reached 60 per cent. of its maximum size, it begins to lose this function and increases in importance as an exporting organ. The destination of material moving from mature leaves is primarily the

---

* Botany School, University of Melbourne; present address: Farm Lane, Send, Woking, Surrey, England.

†Botany School, University of Melbourne.

shoot apex and the root. It is predominantly the apex when the source leaf is close to the apex, predominantly the root as growth takes the apex further from the source leaf.

In general, it is concluded that the radioactive compound moves along a concentration gradient of *the whole assimilate*, labelled and unlabelled. It does not move uniformly along its own concentration gradient.

I. INTRODUCTION

This paper is concerned with the movement of compounds containing ^{14}C, produced in photosynthesis by the soybean (*Glycine max.* (L.) Merr.). Emphasis has been placed on the investigation of the spatial distribution of assimilate in plants bearing several mature trifoliate leaves, and on changes in the pattern of distribution when the plant is subjected to treatment such as partial defoliation or masking from light.

II. METHODS

Plants of soybean (cv. Biloxi in most experiments) were used because the large thin leaves and long internodes are satisfactory for radioautography. They were grown in pots in a greenhouse under variable environmental conditions. The labelled assimilate was produced by allowing part of a leaf to photosynthesize in an atmosphere containing $^{14}CO_2$. The isotope was supplied as barium carbonate from the Radiochemical Centre, Amersham, England, appropriate mixtures being obtained by further dilution with inactive barium carbonate. Great care was exercised to obtain a uniform mixture. The carbonates were mixed in small amounts at a time, and ground together with a glass rod on a ground-glass surface; the final mixture was given prolonged grinding in a mortar and pestle and samples of 1 mg checked for similarity by counting. The specific activity of the mixture used is stated for each experiment.

The carbon dioxide was usually supplied locally to part of a leaflet only, in an assimilation chamber made from a 10-ml weighing bottle with an open end usually of cross-sectional area 1 sq. cm. The initial carbon dioxide concentration in the assimilation chambers was calculated to be 1 per cent. A 1-mg sample was weighed into a chamber after the ground edges of the open end had been coated with porometer luting wax (a mixture of colophony resin, "Vaseline", and small pieces of rubber tubing, heated to melting, stirred, and cooled). The chamber was held in a clamp just under the leaf surface.

An excess of 1N HCl was delivered to the bottom of the exposure chamber with a hypodermic syringe and the abaxial side of the leaf immediately sealed to the exposure chamber. The whole plant (with the exception of any masked region) was exposed to sunlight for a specified period, usually either 1 or 2 hr. On dull days the daylight was supplemented by light from a bank of white fluorescent discharge tubes. In most experiments the intensity ranged from 500 to 2000 f.c. Under these conditions it was shown (by transferring the chamber from leaf to leaf at intervals of 20 min) that most of the $^{14}CO_2$ in the chamber was lost in 40 min, and that insignificant amounts remained after 2–2½ hr.

It was necessary to find a method of tissue drying which would minimize the movement of assimilate after the experiment proper had ceased. Preliminary tests showed that freeze-drying is a satisfactory method when the detailed distribution of isotope within a single organ is required. It is, however, inconvenient for experiments using many large plants, and it often leads to distortion of the leaves. Even when light pressure is applied to the leaf during freeze-drying, the distortion is such that intimate contact of a leaf with the X-ray film is not possible. For the purposes of this work we relied mainly on the immediate dissection of the plant, after its illumination, into suitable pieces (e.g. half leaves, leaf segments, internodes) and the air-drying of the separate pieces between clean sheets of absorbent paper maintained under light pressure. This method is simple and satisfactory for the study of the movement of assimilate from one organ to another, or movement within an organ which is dissected before drying (Thaine and Walters 1955).

In the preparation of radioautographs the segments of dried leaves, stems, and roots were placed on sheets of paper 5 by 7 in., their outlines traced, and other identifying details noted. The paper with the specimens was then placed between two sheets of $\frac{1}{8}$-in. glass of the same dimensions. Finally, in the darkroom, using a No. 6B Wratten filter, a piece of "Kodirex" (no-screen) duplitized X-ray film was placed in contact with the tissue and the whole held firmly with spring-back clips. These plates were kept at 1°C for 14 days in a light-tight tin. Development was with the manufacturers' developer for 3 min at 20°C, and an acid fixing solution was used. Contact prints were made from the radioautographs, using lightweight glossy paper of high contrast, exposed for 1–3 sec, 5 ft from an unshaded frosted 300-W incandescent globe. With perfectly dry clean tissue no difficulty was experienced from pseudophotographic effects.

The absorbent paper used for drying was checked for activity by placing it in contact with X-ray film for 14 days. In no case could any activity be shown to have moved from the active soybean tissue into the drying paper.

In some experiments we have supplemented the information given by radioautographs by measuring radioactivity by means of a Geiger–Muller counter and scaler. For the experiments described here we have not measured total or soluble radioactive material in any tissue. Counts have been made on known areas of thin dried tissue. Owing to self-absorption, the values obtained are assumed to give only the same information as would accurate measurement of the density of the radioautograph. They thus give quantitative expression to the radioautograph and allow tabling and the statistical treatment of the results.

The vascular anatomy of the soybean stem and petiole was studied by serial hand-sections, examined fresh, and stained.

III. The Vascular Anatomy of the Soybean

All the leaves in the soybean plant are linked by vascular connections. Transverse sections of the stem show that, whilst there are usually about 10 xylem bundles, there are 50–55 small phloem strands fairly evenly spaced around the stem. Longitudinal sections of the stem show these phloem strands to be connected by numbers of sieve-tube bridges passing diagonally between them. Sections of the petiole show a similar construction throughout most of its length. In the pulvinar region, the vascular strands of the petiole come together to form one large central contracted bundle, the outer part of which is a continuous annulus of phloem tissue. This bundle divides into three and the three petiolar strands enter the axis stele at three points, one immediately opposite the petiole and the other two at points 90° to each side of it.

Thus, in the stem the phloem tissue does not run in strictly defined longitudinal bundles, but rather is a loose anastomosing network of sieve-tube strands, the three strands of petiolar phloem entering this network at three points well separated on its circumference.

IV. Results and Conclusions

(a) *The Redistribution of Labelled Translocate in Whole Plants*

When part of a green leaf is exposed to light, for at least 1 hr, in the presence of $^{14}CO_2$ (specific activity 0·6 mc/m-mole), the radioautograph of the site of application appears in the negative as a black circle or square with a diffuse margin (c.f. Plate 2, Fig. 3). A considerable proportion of the labelled material is fixed at the site as immobile compounds, but some of it is mobile in the veins and moves out of the leaf during the experiment. Movement within the source leaf will be discussed later. During the course of photosynthesis for 1 hr, isotope moves from its site of application to the root tips, stem, apex, and youngest unfolded leaves. In the growing organs (stem and root tips and young leaves), the translocate appears to enter all the cells. In the mature source leaf, the stem, and the root it is located mainly in the veins, which usually show clearly on the radioautograph. However, there is always some movement from the veins to the surrounding tissue during air drying. Sometimes the radioautographs also show an accumulation of isotope at the cut ends of the veins, indicating some mass flow of material on cutting the phloem or during the early stages of drying.

(i) *Experiment* I-1 (*specific activity* 0·6 *mc/m-mole*).—Eight soybean plants were used, each with two fully expanded trifoliate leaves. $^{14}CO_2$ was applied to the uppermost fully expanded leaf, in four cases to the terminal leaflet, and in four cases to one or other of the lateral leaflets (Fig. 1). The period of exposure was 2 hr, the light intensity 700–1000 f.c. At the end of the exposure plants were immediately dissected, air dried, and radioautographed. This experiment was repeated twice, and for a total of 24 plants the radioautographs showed an identical pattern of distribution (Plate 1). The labelled assimilate moved to the apex and to the root (where it was concentrated in the root apices) and to the immature leaf above the source leaf, but not (in amounts necessary to produce a radioautograph) to the primary leaves or to the fully developed trifoliate leaves, or to the other leaflets of the source leaf. It is concluded that, for the purposes of these experiments, it is immaterial which leaflet of a source leaf acts as the site of $^{14}CO_2$ application.

During the course of the experiments described in later sections of this paper it became apparent that the age, size, and position of each source leaf in relation to apex and root were important factors concerned in the direction of the movement of labelled assimilate.

The pattern of redistribution of assimilate in whole undamaged plants was clarified by the results of the following experiment.

(ii) *Experiment* I-2 (*specific activity* 0·6 *mc/m-mole*).—Twelve similar soybean plants were used (Fig. 1, *A–D*). Each carried two simple primary leaves (1), two fully expanded trifoliate leaves (2, 3), and one expanding leaf (4), whose length varied between 20 and 90 per cent. of the mean adult leaf length. The apex and each type of leaf served as application sites for $^{14}CO_2$ and each site (including the apex) subsequently gave dense radioautographs. It was necessary to use an exposure chamber of modified shape for application of the gas to the apex. Exposure to $^{14}CO_2$ was for 2 hr at a light intensity of 2000 f.c., the remainder of each plant being similarly illuminated in air. At the end of the exposure period the plants were dissected, air dried, and radioautographed. The results are summarized in Table 1.

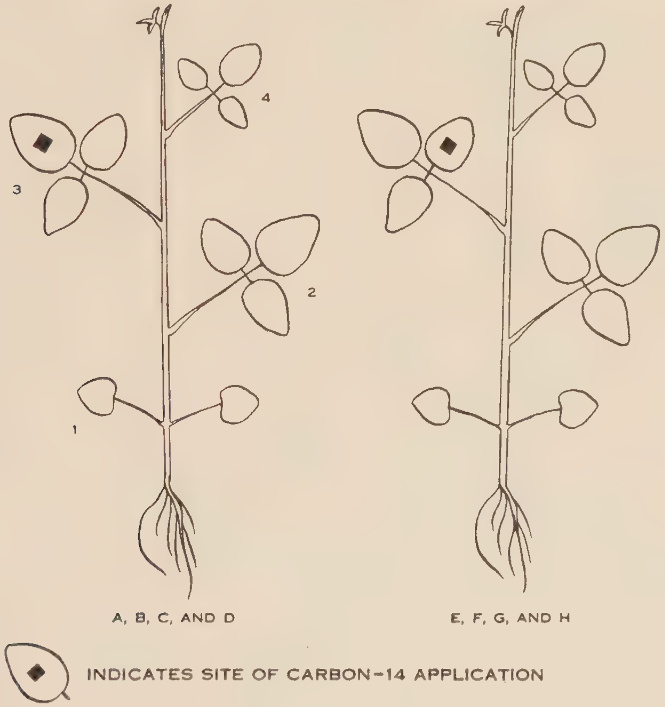


Fig. 1.—Plan of experiment I-1, with four plants in each group. Plants similar to these also used in experiment I-2.

These clear but qualitative results were supplemented, in the same experiment, by counts made on approximately equal areas (1 in. sq.) of the dried plant tissue taken from different organs. The values obtained showed that the total amount of labelled assimilate formed and translocated under the same conditions varied according to the age of the tissue used as the site of application (Table 2(*a*)).

Distribution of the translocate between different parts of the plants was, therefore, calculated as a percentage of the total translocate for each plant (Table 2(*b*)). The results support those summarized in Table 1, and serve to indicate still more clearly the pattern of distribution.

The results of this experiment are substantiated by those of numerous other experiments carried out with different objects in view. Clearly, the radioactive fraction of the assimilate does not diffuse through the plant along its own concentration gradients. If we make the assumption that it acts as a tracer for the untagged assimilates, the results lead to the following conclusions.

TABLE 1

EXPERIMENT I-2: THE REDISTRIBUTION OF LABELLED ASSIMILATE IN WHOLE SOYBEAN PLANTS

Arbitrary scale of densities on radioautograph: +++++, very dense; ++++, dense; +++, medium; ++, faint; +, very faint; 0, nil

Plants	Leaf No. (site of $^{14}CO_2$ application, see Fig. 1)	Comparative Density of Radioautograph in:						
		Root	Leaf 1	Leaf 2	Leaf 3	Leaf 4	Degree of Expansion of Leaf 4 (%)	Apex with Apical Leaves
A	1	+++++	(source)	0	0	+	85	+
B	1	+++++	(source)	0	0	+	85	+
C	2	++++	0	(source)	0	++	80	++++
D	2	++++	0	(source)	0	++++	50	++++
E	3	++++	0	0	(source)	++++	60	++++
F	3	++++	0	0	(source)	++++	60	++++
G	4	+++	0	0	0	(source)	90	++++
H	4	0	0	0	0	(source)	65	++
I	4	0	0	0	0	(source)	60	0
J	4	0	0	0	0	(source)	45	0
K	Apex with apical leaves	0	0	0	0	0	20	(source)
L	Apex with apical leaves	0	0	0	0	0	20	(source)

For the lowermost leaves the major "sink" for assimilation is the root; for the uppermost leaves it is the apex and young expanding leaves. Leaves in a median position supply both apex and root. The apex and young expanding leaf of below 60 per cent. adult length show no export of active assimilate, the expanding leaf of greater size exports an increasing amount of assimilate, primarily to the apex.

TABLE 2

RESULTS OF EXPERIMENT I-2 AND COMPARISON WITH OTHER DATA

(*a*) Relative amounts of total translocate provided by leaves of different age and type (see Fig. 1). The values are based on mean counts per min in all organs other than the source leaf

Leaf 1	Leaf 2	Leaf 3	Leaf 4: Degree of Expansion			
			90%	65%	60%	45%
33	90	100	85	7	0·5	0

(*b*) The distribution of labelled assimilate in the whole plant (see Fig. 1)

Leaf No. (site of application of $^{14}CO_2$)	Percentage of Total Translocate Counted Found in:				
	Root	Leaf 4 (expanding)	Apex	Leaf 4 + Apex	Mature Leaves
1	92	5	3	8	0
1	88	10	2	12	0
2	63	4	33	37	0
2	45	14	41	55	0
3	31	9	60	69	0
3	37	42	21	63	0
4	14	(source)	86	—	0
4	9	(source)	91	—	0

(*c*) The distribution of labelled assimilate in the whole plant of soybean (data of Belikov 1958*a*). Values are ^{14}C activity of plant part (counts/min/disk) after photosynthesis for 20 min expressed as percentage of total assimilate

Plant Part	Mature Source Leaf:			
	Leaf 1 (primary)	Leaf 2 (oldest trifoliate)	Leaf 3	Leaf 4
Stem above source leaf	20	27	47	57
Stem and root below source leaf	80	73	53	43

Thus, a leaf, in passing from its position as a small folded leaf at the apex, through the stages of expansion to a fully expanded leaf, and possibly (by abscission of lower leaves) to the position of lowest leaf, shows also a steady change in its economy.

As a young leaf, up to 60 per cent. of adult length, it forms part of the major apical sink for upward-moving assimilate and for its own photosynthetic product; it exports nothing to the rest of the plant. Above this size it steadily loses its function as part of the apical sink and increases in importance as an exporting organ. The destination of material moving from such a leaf once it is fully expanded is both apex and root, predominantly the apex when the leaf is in a position close to the apex, predominantly the root as growth takes the apex further from the leaf.

In experiment I-2 there was no evidence of the import of labelled assimilate into mature leaves. Although a major role of the fully expanded leaf is that of an exporting organ, it will later be shown that it may import small amounts of assimilate from other leaves on the plant.

These conclusions are consistent with and supplement other results. Belikov (1955) found that labelled assimilates accumulate in the growing tips of stems and in young leaves of the soybean. Aronoff (1955) stated that for the same species the "movement of labelled photosynthate is greatest to the growing regions." Nelson and Gorham (1957*a*) showed that the labelled photosynthate produced in the *primary* leaf moved mainly downwards towards the roots. Vernon and Aronoff (1952) applied $^{14}CO_2$ to the oldest trifoliate leaf of soybean and found that, while it moved both to apex and base, "the predominance of activity moved basipetally."

Crafts (1956) states that, in cotton and *Cucurbita*, the upper leaves exported applied radioactive 2,4-D mainly to the apex, the lower leaves mainly to the base (roots). He assumed (without direct evidence) that this was also the pattern of redistribution of the assimilates. In earlier work, Goodall (1946) measured dry weight changes in tomato leaves, attached and excised. The youngest leaves provided little assimilate and acted as sinks for material coming from the older leaves. Translocation went on during photosynthesis; and in summer one-half of the translocate from the older leaves moved to the root and only one-eighth to the youngest leaves. In his experiments all the leaves were exposed to a CO_2 concentration of 0·03 per cent. In our experiment the site of application received much higher CO_2 concentration than did the rest of the plant. The general similarity of the two results suggests that the technique we used was not responsible for the pattern found.

In work published after the completion of our own experiments, Belikov (1958*a*, 1958*b*) also applied $^{14}CO_2$ to successive leaves of soybean plants and subsequently measured the radioactivity in parts above and below the source leaf. His results are summarized in Table 2(*c*). His conclusions are closely similar to ours, that the distribution of assimilate is localized, that mature leaves do not import, that from the upper leaves assimilate moves to the apex and young leaves, from the lower leaves it moves to the root, from the middle leaves it moves both up to the apex and down to the root.

These results all indicate the complexity of the problem of translocation in whole plants, and the need for the close matching of replicate plants used for experiments with radiocarbon.

(*b*) *Transport to the Apical Bud*

Experiments on the translocation of food materials from mature leaves to the apical bud seldom appear in the literature of translocation. Nevertheless, such movement must be one of the major pathways of the assimilated compounds. Our experiments have shown that when $^{14}CO_2$ is supplied in the light to a mature leaf of a normal soybean plant, tagged assimilate moves rapidly to the apical bud. We assume that such leaves, when illuminated normally in air, supply carbohydrate to the apex, which also presumably synthesizes additional carbohydrate in its own green cells. The movement of tagged assimilate to the apex was, therefore, investigated under conditions in which the green apical bud was either masked or illuminated, and in plants in which the leaves between apical bud and source leaf were either masked or removed before the experiment began. All three treatments (masking of apex, masking of leaves, removal of leaves) could be expected to lower the concentration of untagged carbohydrate in the apical region.

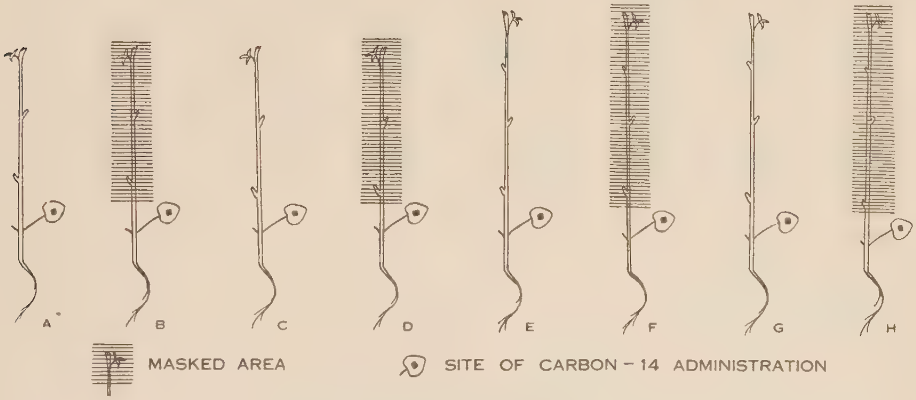


Fig. 2.—Plan of experiment II-2.

(i) *Experiment* II-1 (*specific activity* 0·04 *mc/m-mole*).—In this preliminary experiment, with only two plants, visual inspection of the apex radioautographs suggested that masking the apical region of a defoliated plant (as in Fig. 2, *B*) reduced rather than increased the import of tagged assimilate into the apex. Further experiments were designed to investigate this unexpected result.

(ii) *Experiment* II-2 (*specific activity* 0·04 *mc/m-mole*).—Eight soybean plants were used (Fig. 2), four with two fully expanded trifoliate leaves (*A*, *B*, *C*, *D*) and four with two fully expanded and one expanding leaf (*E*, *F*, *G*, *H*). In each group the plants were carefully selected so as to have apical leaves of uniform size. The plants were defoliated immediately before the exposure to $^{14}CO_2$ began, leaving only the apical bud and one primary leaf. Four plants (*B*, *D*, *F*, *H*) were masked above the primary leaf, the mask covering stem and apex, as shown in Figure 2. Exposure to $^{14}CO_2$ began at 3 p.m. and ended at 5 p.m. Light intensity was approximately

600 f.c. The application sites on the radioautographs showed uniformly high activity, much of which was probably due to radioactive starch and other insoluble materials. As might be expected from the position of the leaf of application, comparatively small amounts of tagged isotope moved to the apical parts of the stems. Radioautographs of the apices and of the upper parts of the stem clearly confirmed the result of experiment II-1. Those of the illuminated apices and stems gave much clearer radioautographs than those in which these parts had been masked from light during the experiment (e.g. see Plate 2, Fig. 3).

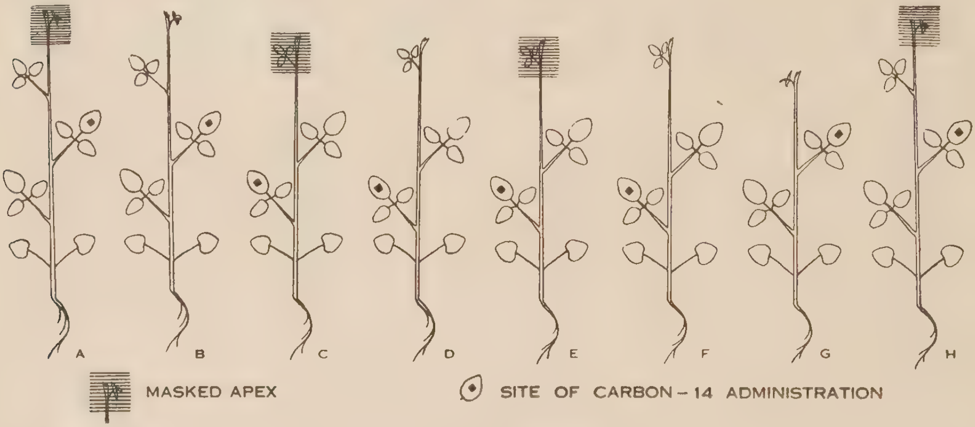


Fig. 3.—Plan of experiment II-4.

(iii) *Experiments* II-3, II-4, and II-5 (*specific activity* 0·04 *mc/m-mole*).—In these three experiments the leaves between apex and source leaf were neither removed nor masked. In all there were 20 plants, and in half of them the apical bud was masked. Figure 3 shows the plan of experiment II-4. In all 20 plants (except Fig. 3, *G*) there was at least one expanded trifoliate leaf remaining between the site of $^{14}CO_2$ application and the stem apex. The application sites were on one or other of the two oldest trifoliate leaves. No consistent differences were found in the intensities of the radioautographs of the 20 apices.

(iv) *Experiments* II-6, II-7 (*specific activity* 0·04 *mc/m-mole*).—In experiment II-6 there were eight plants, each originally with two fully expanded trifoliate leaves. In all of these the application site was a primary leaf. In one group (A) the four plants were normal, in group B they were defoliated except for the expanding leaf and the source leaf. Immediately prior to the period of exposure to $^{14}CO_2$ (3 hr) three plants from each group were apically masked.

Experiment II-7 was a repetition of II-6, except that all eight plants originally had only one fully expanded trifoliate leaf, and the exposure period was for 2 hr. Light intensity in both experiments was approximately 700 f.c.

Comparison of the radioautographs obtained in both experiments showed that the removal of the fully expanded leaves undoubtedly increased the amount of tagged assimilate moving into both the stem and the apex from the site of application. In those plants with all the leaves attached, masking of the apex caused no detectable change in the import to the apex. This result confirmed those of experiments II-3, II-4, and II-5. On the other hand, a clear confirmation of experiments II-1 and II-2 was not obtained. In defoliated plants, masking of the apex clearly decreased import to the apex in experiment II-6, but in II-7 the differences in the radioautographs were too slight to allow any distinction to be drawn between them.

(v) *Experiment* II-8 (*specific activity* 0·04 *mc/m-mole*).—Seventy-two soybean plants were used, each of which had three fully expanded trifoliate leaves. The experiment was carried out in a greenhouse over a period of 9 days, at a mean light intensity of 1500 f.c. The plants were divided into three groups (L_i, L_m, and L_o), and for convenience of operation, eight members of two of these groups were dealt with at one time in a so-called "block". There were nine blocks, each consisting of eight plants arranged in pairs. The treatments are set out in Table 4. The $^{14}CO_2$ was applied for 3 hr to the lowest trifoliate leaf of each plant in a block. In groups L_m and L_o, the two mature leaves between source leaf and apex were masked from the light (L_m) or cut off (L_o) 24 hr prior to the application of the $^{14}CO_2$. Masking was continued during the 3 hr of the experiment. The apices were masked 1 hr prior to and during the assimilation of the $^{14}CO_2$. At the end of the 3-hr period the apices were cut off, air dried under pressure, radioautographed, and the radioactivity counted. The results are set out in Tables 3 and 4. The results were submitted to a statistical analysis (see Appendix I).

Table 3

EXPERIMENT II-8: RADIOAUTOGRAPHY OF STEM TIPS

No. of Plants in each Experimental Group	Percentage of Plants giving Apical Prints with:				
	Leaves Present and Illuminated	Leaves Present but Masked	Leaves Cut Off	Apices Illuminated	Apices Masked
24	25	75	100		
36				72	
36					61

(vi) *Experiments* II-1–II-8: *Conclusions*.—In experiment II-8, the radioautographs (Table 3) and the mean apical counts of ^{14}C (Table 4) for groups L_i (all leaves attached and illuminated) and L_o suggest that the removal of the mature leaves, between source leaf and apex, results in a greatly increased movement of the tagged assimilate to the apex. There were considerable variations in the counts; nevertheless, the analysis of variance showed that this effect is highly significant (see Appendix I). It was concluded that the removal of the mature leaves increased the amount of radioactivity in the apex by about 60 per cent. This result confirms that obtained qualitatively in experiments II-6 and II-7.

Mature illuminated leaves have been shown to import negligible quantities of ^{14}C from a source leaf under the conditions of this experiment. Hence the results just described cannot be due to the receipt by the apex of ^{14}C which was prevented from entering the mature leaves because these were excised. We have adopted the hypothesis that the increased export of ^{14}C to the apex was due to the lowering of the concentration of *total* assimilate in the apex, consequent upon the removal of

TABLE 4

PLAN AND RESULTS OF EXPERIMENT II-8

Values given are apical counts of ^{14}C, expressed as counts/minute corrected for background

Block No.	Group L_i*		Group L_m†		Group L_o‡	
	Apex Masked	Apex Illuminated	Apex Masked	Apex Illuminated	Apex Masked	Apex Illuminated
1	19	11			11	15
	2	0			24	5
2	0	2			4	6
	2	0			2	4
3	0	0			6	22
	6	4			8	2
4			6	12	28	48
			2	22	36	26
5			26	18	24	16
			0	0	36	16
6			2	0	14	6
			0	6	0	40
7	6	28	28	22		
	4	4	2	52		
8	8	8	10	18		
	4	12	10	20		
9	6	6	44	12		
	2	2	4	18		
Mean all plants	4·9	6·4	11·2	16·7	16·1	17·2
	5·7		14·0		16·6	

*L_i, all leaves attached and illuminated.

†L_m, all leaves attached, upper two (between source leaf and apex) masked.

‡L_o, upper two leaves (between source leaf and apex) removed.

the two mature leaves. For in the control plant such leaves, illuminated in air, would be expected to export untagged assimilate to the apex.

In experiment II-8 (Table 4) the means of counts for groups L_i and L_m suggested at first that masking of the leaves between source and apex also increased movement to the apex. However, the differences, although large, did not reach statistical significance. One would expect masking to have a similar effect to defoliation, but it may be that the effects of masking are two-fold and counterbalancing. While the darkened leaf (like the excised one) could export little assimilate to the apex, the possibility exists (see Section III*c*) that it could import ^{14}C from the source and thus divert it from the apex.

If the hypothesis set out above is correct, then one would expect that masking of the green apex alone would increase its import of ^{14}C from the source leaf. No such increase has been detected. Rather the sum total of numerous experiments suggests the opposite—that masking the apex *decreases* the import. Qualitative support for this was given by the radioautographs of experiments II-1, II-2, II-6, and II-8 (but not II-7), in plants of which the leaves between source and apex were removed. On the other hand, in experiments II-3, II-6, II-7, with all leaves present, there was no such decrease due to masking. Finally, when the matter was put to quantitative test (expt. II-8, Table 4), while the mean counts were smaller for the apically masked plants in all three groups, the differences were not statistically significant. It appears that the apical masking effect, if present at all, is too small to be detected except possibly by precise counting methods applied to a simplified biological system, i.e. largely defoliated plants in which downward movement of assimilate to the root is prevented. Vernon and Aronoff (1952) have reported that light has no direct effect on translocation. Nelson and Gorham (1957*a*) have noted that a smaller amount of ^{14}C is transported to the stem apex in the dark than in the light, but in their single experiment the whole plant was darkened after a 15-min light period. Moreover, the two plants used were not evenly matched.

(*c*) *Translocation in the Leaf*

(i) *Export from Masked Leaves*

Two experiments were designed to test the assumption that leaf masking decreases the concentration of the mobile products of photosynthesis in the leaves and in the phloem of the leaf veins.

(1) *Experiment* III-1 (*specific activity* 0·04 *mc/m-mole*).—Eight similar plants were selected, each with two fully expanded trifoliate leaves. On each the terminal leaflet of the first leaf was masked for 24 hr. It was then illuminated for 3 hr, at 300 f.c. and while the $^{14}CO_2$ was being applied to a central area. The exposure chambers were then removed and the leaves immediately masked again. Leaves were harvested at 0, 1, 2, 3, 4, 5, and 6 hr from this second masking. Each leaflet was severed from its petiole and cut transversely just below the site of application of $^{14}CO_2$. The upper segment was then air dried and radioautographed. In Plate 2, Figure 1, the appearance of the eight central brighter areas (application sites) gives us no information about total export during the second masking. There is no

regular decrease in the activity with time—possibly there was uneven uptake. On the other hand, there is radioactivity in the veins passing out from the central areas in all eight figures. Although this was weaker in the samples masked for 6 hr, it is clear that the export of readily mobile material was not complete even after this period.

(2) *Experiment* III-2 (*specific activity* 0·04 *mc/m-mole*).—The procedure was similar to that described above, but the photosynthetic period was 2 hr at 500 f.c. and a longer period was used for the second masking, leaves from two plants being harvested at 4, 24, 48, and 72 hr. Before air drying, each leaflet was cut transversely into three segments and in Plate 2, Figure 2, the radioautographs of the central segments only are shown.

Lateral spread of the isotope was detectable (Plate 2, Fig. 2) in radioautographs of leaves harvested after masking for 4 hr. There was no activity in the peripheral veins of the leaf segments harvested after masking for 24 hr or longer, this suggesting that, after this period, the readily mobile or mobilizable products of a 2-hr photosynthesis period had been exported from the application site. This conclusion is valid even if some of the movement takes place during air drying, and in spite of the fact that the activity of the central area (site of application) showed no consistent decrease with time. The essential features that emerge from these two experiments are that (*a*) in leaf segments darkened for 24–72 hr and still with high radioactivity at the site, there is no activity detectable by radioautography in the veins leading from this site; (*b*) there is such activity in the peripheral veins of such segments darkened for only 0–6 hr. We know from iodine tests of this and parallel material that starch is all lost from the site after darkening for 24 hr. It may, therefore, be presumed that the activity shown in the central areas of the leaflets darkened for 24–72 hr is from relatively insoluble (or immobile) material which is not starch.

It is concluded that the concentration of readily mobile assimilate in the veins of a soybean leaf masked for 24 hr is lower than the concentration in the veins of an illuminated part; the magnitude of the difference is unknown.

(ii) *Transport into Leaves*

Carbohydrates synthesized in mature leaves pass from the leaf to the regions of growth and development. If the movement is necessarily along a positive concentration gradient in the phloem, reversal of the normal gradient might be expected to cause reversal of movement, e.g. import into a mature leaf. The question of import by mature leaves is relevant in any consideration of translocation theory and the results of a preliminary study of it are now presented.

(1) *Experiment* III-3 (*specific activity* 0·04 *mc/m-mole*).—Six soybean plants (*A*–*F*) were prepared according to the diagram (Fig. 4). The plants had four fully expanded trifoliate leaves and one very small expanding leaf. In plants *A*, *B*, and *E*, these young leaves, together with the stem apices, were removed just prior to the application of the $^{14}CO_2$. This eliminated the major sink for material coming from the uppermost mature leaf, which was the source. In *B*, *D*, *E*, and *F*, all the

other leaves were masked for 48 hr before the application of the $^{14}CO_2$ (light intensity *c.* 500 f.c. for 4 hr). All leaves were harvested separately after another 20 hr had elapsed, so that the total time of shading was 72 hr.

Radioautographs of the harvested leaves showed that in five of these plants (*E* was damaged during the experiment) there was no detectable import of tagged assimilate into either masked or illuminated fully expanded leaves, irrespective of the presence or absence of the apex and expanding leaf. Judging visually from the radioautographs, it could be assumed that the amount of $^{14}CO_2$ fixation was relatively uniform in the five plants, and in *A* and *B* removal of the apical parts of the stem led to an increased transport of tagged assimilate to the root. There was no ^{14}C activity detectable in either the mesophyll cells or the veins of the harvested "receptor" leaves of any of these plants. Nor was there any activity detectable in the lateral (illuminated) leaflets of the "source" leaf.

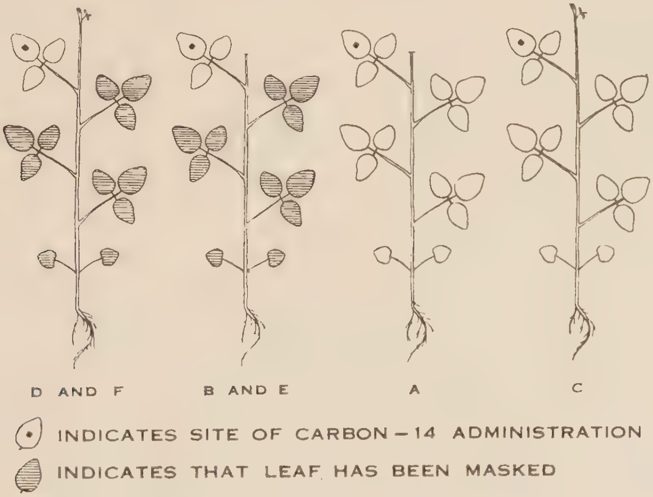

D AND F
B AND E
A
C
INDICATES SITE OF CARBON – 14 ADMINISTRATION
INDICATES THAT LEAF HAS BEEN MASKED

Fig. 4.—Plan of experiment III-3.

This result is typical for experiments of this type, in which the specific activity at the source was low (0·04 mc/m-mole). In experiment I-1 (Plate 1), no leaves were masked, but 25 plants have been used in experiments in which mature leaves (usually two) were masked for 24 hr, the $^{14}CO_2$ being applied to another mature leaf for the last 2 hr of this period. None of the masked leaves gave a radioautograph, whereas in all cases there was very marked activity around the $^{14}CO_2$ application site, and tagged assimilate was shown to move to the apex and expanding leaf (if present) and to the main stem and root.

The apparent failure of these mature leaves to import assimilate could have been due to the absence of vascular connection between the illuminated leaf and the masked leaves. This was considered improbable for the following reasons:

(*a*) The movement of tagged assimilate from mature leaves to young expanding leaves (e.g. expt. I-1). The translocation pathway thus demonstrated (and presumed to be phloem) is not likely to be broken as the leaf matures, for mature leaves can all *export* assimilate to the stem, apex, and root.

(*b*) The lack of import shown by all five masked leaves of plants such as Figure 4, *D*; at least one of these could be expected to be linked via the phloem to the exporting leaf.

(*c*) Experiments with aqueous eosin showing that there are vascular (xylary) connections between all the leaves of a plant with three fully expanded trifoliate leaves. Regardless of which leaf was cut under eosin solution, the dye moved in the xylem to the veins of all other leaves.

(*d*) The anatomical peculiarities of the phloem tissue in the soybean, briefly described in Section III.

In the experiments already described, the specific activity of the barium carbonate used was low (0·04 mc/m-mole). It was adequate to demonstrate movement of assimilate from the leaf to the veins of the stem, to the root, the apex, and to leaves which had expanded to less than 85 per cent. of their full size. If there is some movement to the mature leaves, it should be demonstrable by raising the activity at the source. In the following experiment this was tested by using barium carbonate mixtures of three different specific activities, the total carbon dioxide concentration in the exposure chamber remaining at 1 per cent. in each case.

(2) *Experiment* III-4 (*specific activities* 0·04, 0·6, *and* 17 *mc/m-mole*).—Twelve plants were used, each having two mature trifoliate leaves and two primary leaves, the $^{14}CO_2$ being applied to a leaflet of a mature trifoliate leaf. No masking treatment was given these plants, and the light intensity was 700 f.c. The plants were dissected at the end of the experiment and the whole plant radioautographed. The radioautographs of source leaflet, apex, root, and stems showed increasing density with the increasing specific activity at the source; those with the highest activity were overexposed.

No indication of activity in the mature leaves could be seen in the group which had received the $^{14}CO_2$ at the lowest specific activity. In the medium activity group (0·6 mc/m-mole) very faint prints of the lateral leaflets of the source leaf could be seen, and scarcely perceptible prints of the basal parts and midrib in other mature leaves (trifoliate and primary).

In the group receiving the highest activity (17 mc/m-mole), the densest radioautographs were still those of the apex, but all the mature leaves now gave faint prints, the activity being greater in the veins than in the mesophyll (Plate 2, Fig. 4).

The total concentration of carbon dioxide at the source leaf was the same in this as in earlier experiments, and there is no reason to suppose that the import into these mature leaves is via the xylem. The result is consistent with the view already expressed, that there is phloem connection between individual leaves in the soybean. The extent of import into mature leaves now demonstrated is still small in comparison with that into apex or root. The radioautograph of Plate 2, Figure 4, suggests that the tagged material moves not only into veins, but also into the mesophyll cells of mature leaves. This latter, however, could be movement during air drying. Clarification of this aspect requires more refined methods (e.g. those of Thaine and Walters 1955).

(iii) *Movement within Single Leaves: Leaves Attached to Whole Plants*

(1) *Experiment* III-5 (*specific activity* 0·04 *mc/m-mole*).—The plan and results of this experiment are shown in Figure 5. Two growing plants were used and certain leaf areas were masked for 24 hr. The $^{14}CO_2$ was then applied for 4 hr in the usual way to each of three locations on plant A and five locations on plant B. During this period the unmasked parts were illuminated in sunlight (approx. 500 f.c.). Leaves or leaflets bearing masks were then excised, freeze-dried, and radioautographed. In this experiment there was considerable distortion of the leaves during freeze-drying, and diagrams are used instead of photographs to summarize the results.

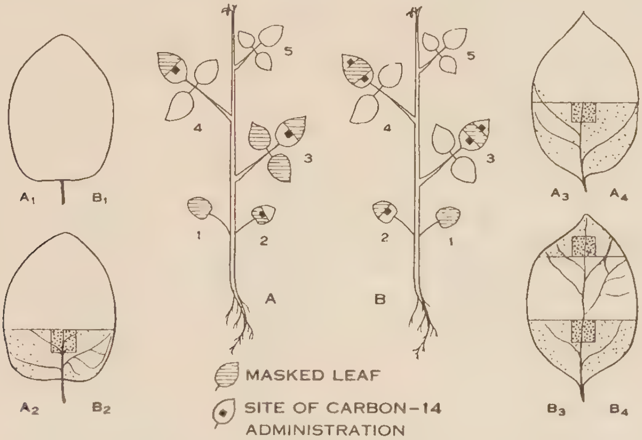


Fig. 5.—Plan and result of experiment III-5. The larger leaf diagrams are drawn from the radioautographs obtained after freeze-drying. Dark lines (other than leaf outlines) and dots indicate activity.

As Figure 5 shows, under conditions in which there is always extensive movement from the source leaves to roots and stem apices, there was no movement of tagged assimilate into the masked primary leaves A_1, B_1, or into the upper halves of masked leaves A_2, B_2, A_3, A_4. In plant B, such movement as occurred into the central masked area appeared to be from the apical application site downwards towards the petiole. The outer margins of the masked areas were sharp, indicating that movement into these areas, if it occurred, was via the veins, not the mesophyll cells. On the other hand, the application sites within the illuminated areas were not so clearly defined, and we assume that this was due to some photosynthesis of $^{14}CO_2$ diffusing outwards in the leaf from the site of application (see expt. III-7).

(2) *Experiment* III-6 (*specific activity* 0·04 *mc/m-mole*).—This experiment was similar to III-5, but the $^{14}CO_2$ was applied to one leaf only (as in Fig. 5, A_4 only), just below the masked area. Total period of masking was 49 hr, the $^{14}CO_2$ being applied for the last 3 hr of this period at a light intensity of 500 f.c. The leaves were air dried after having been cut into halves along the line of the masks and then

severed from their petioles. Typical radioautographs of two of five plants used are shown in Plate 3, Figures 1 and 2.

In plant *A* (Plate 3, Fig. 1), in which all the leaves were green and healthy, there was no movement of isotopic material into the unmasked primary leaves, the unmasked lateral leaflets, or into the masked upper half of the source leaf. This was in agreement with experiment III-5. In plant *B* (Plate 3, Fig. 2) again there was no movement into the unmasked primary leaves, and very little into the unmasked lateral leaflets (the slight basal activity here could have been due to leakage of $^{14}CO_2$ from the nearby source). However, there was in the terminal-source leaflet marked acropetal movement into the masked apical half and accumulation of activity around small chlorotic spots, believed to be caused by nutrient imbalance in the potting soil.

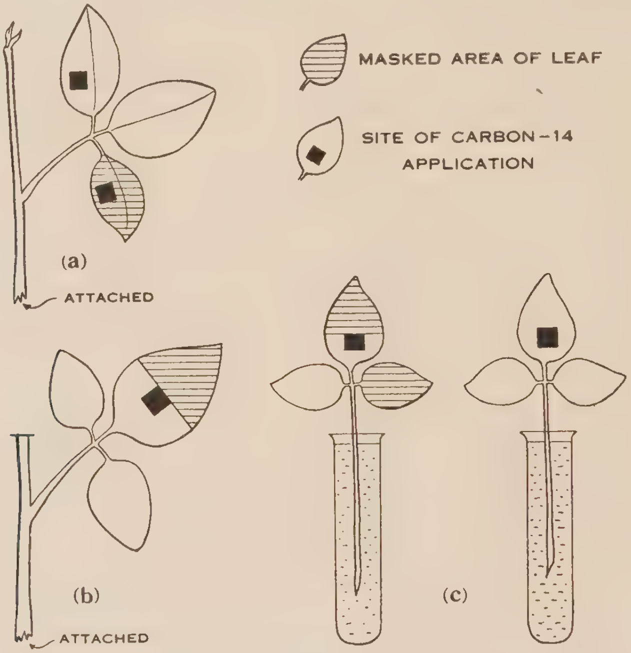


Fig. 6.—(*a*) Plan of experiment III-7 showing only attached source leaf. (*b*) Plan of experiment III-8 showing attached source leaf with mask and main stem with apex removed. (*c*) Plan of experiments III-9–III-14, showing detached leaves with petioles in water.

(3) *Experiment* III-7 (*specific activity* 1·06 *mc/m-mole*).—Eight healthy, mature, attached leaves (on three plants) were used. The specific activity at the source was high and the experiment was done on a bright sunny day in midsummer (light intensity 2000 f.c.). The application sites were on opposite lateral leaflets, one fully illuminated, the other completely masked except for the application site (Fig. 6(*a*)). Four leaves were dried before, and four after, being cut into segments transversely across the edges of the application site.

Unless the leaf is both masked as shown and segmented before drying, the radioautographs give a misleading picture of translocation, suggesting that the tagged assimilate moves both acropetally and basipetally to the same extent, and also crosses the main vein at least in the distal parts. A great deal of this movement, however, takes place during air drying (and even freeze drying), while a considerable part of the activity outside the application area proved to be due to photosynthesis of $^{14}CO_2$ diffusing from this area into the rest of the leaf. In the only radioautograph shown (Plate 3, Fig. 3) both artefacts have been avoided (by masking and cutting) and the major movement has clearly been basipetal in the major veins. Movement across the main vein was inconsiderable. There was still considerable lateral movement during air drying in half of the central sector.

(4) *Experiment* III-8 (*specific activity* 0·04 *mc/m-mole*).—Six large plants were used, each with five fully developed trifoliate leaves, all of which were illuminated in air during application of $^{14}CO_2$ to the apical leaflet of the youngest mature leaf. The site was central (Fig. 6(*b*)) or lateral, and the leaflet was masked in its upper half. In addition, the stem apex was removed 48 hr before the beginning of the experiment. Light intensity was high, 1000 f.c., and the period of $^{14}CO_2$ application was 4 hr. Under these conditions it was considered that the gradient of mobile assimilate between the lower and the masked halves of the terminal leaflet would be steeper than in the experiments already recorded, although the root still existed as a sink. At the end of the experiment the terminal leaflet was removed and cut into two just above the line of the mask; the parts were air dried, radioautographed, and the result shown in Plate 3, Figure 4, is typical of those obtained from the six plants.

It is clear that under these conditions tagged assimilate moved acropetally into the veins of the masked leaf tip. There was little movement across the main vein into the other half of the leaf.

(iv) *Movement within Single Leaves: Leaves Detached from the Plant*

(1) *Experiments* III-9–III-14 (*specific activity* 0·04 *mc/m-mole*).—In each experiment there were eight trifoliate leaves, four partially masked, four unmasked, as shown in Figure 6(*c*). While still attached they were pre-illuminated in air (except for the masked areas) for periods stated in Table 5. At the end of this period tests showed that the illuminated tissues contained starch, while the masked portions were free of starch. After this pretreatment the petioles were cut under water and the detached leaves were placed with the cut ends of the petioles dipping into water. $^{14}CO_2$ was applied to the lower surface of the lower half of each terminal leaflet, and after 2 hr exposure the leaflets were separated, the terminal leaf cut into two just above the application site (and along the line of the mask when present). The pieces were air dried and radioautographed. Six of these experiments were carried out, the conditions and results being summarized in Table 5.

It is shown, therefore, that under conditions where the normal sinks have been removed, tagged assimilate can move acropetally within a single leaflet and from the apical leaflet into a lateral leaflet of a trifoliate leaf. This latter demonstrated again that there is a phloem connection between the leaflets. Such acropetal movement, however, was only rarely obtained when the importing leaf part was illuminated in air (a total of six cases out of 48, and in these six the movement was slight, the radioautograph being faint). Acropetal movement into the masked apical halves of leaves or into masked leaflets was clearly shown in 27 out of 48 cases.

The considerable variation in the behaviour of comparable samples appears to be characteristic for experiments on translocation. It is known that the contents of the sieve tubes are under pressure: the possibility, therefore, exists that the failure of movement into nearly half the masked lateral leaflets was due to damage to the phloem at the nodal region consequent upon cutting the petiole.

TABLE 5

DETACHED LEAVES OF SOYBEAN: DISTRIBUTION OF LABELLED ASSIMILATE

Expt. No.	Period of Pre-exposure in Air of Unmasked Leaves to Light (hr)	Light Intensity during $^{14}CO_2$ Application (f.c.)	Proportion of Leaflets in each Experiment showing Acropetal Movement of Labelled Assimilate into:			
			Leaflet Tips		Lateral Leaflets	
			Masked	Illuminated	Masked	Illuminated
III- 9	5	1500	4/4	0/4	0/4	0/4
III-10	7	750	4/4	3/4	3/4	0/4
III-11	24	1500	3/4	0/4	2/4	0/4
III-12	24	1000	2/4	1/4	4/4	1/8
III-13	24	1200	2/4	0/4	1/4	1/8
III-14	24	500	2/4	0/4	0/4	0/4
Totals			17/24	4/24	10/24	2/24

(2) *Experiments* III-1–III-14: *Conclusions*.—Following early experiments with whole plants and with $^{14}CO_2$ supplied at low activity, it was concluded that import into mature leaves was negligible (e.g. expts. I-1, III-3, III-5, III-6). A similar conclusion has been drawn by others. Thus, Aronoff (1955) stated that no flow occurs within a leaflet or to an adjacent mature leaflet merely because of a concentration gradient. "Darkening of leaves for 48 hr prior to $^{14}CO_2$ application never has a greater effect than the normal variation". Vernon and Aronoff (1952) could not demonstrate any movement of radiocarbon into either the primary leaf or the cotyledon. Nelson and Gorham (1957*b*) showed that "only a trace of ^{14}C was translocated to the primary leaf opposite to the source leaf". Belikov (1955) also reported that mature leaves do not import, even if they are deficient in assimilates, and again (1958*a*, 1958*b*) that no movement occurs into the shaded part of a source leaf "even although it was dying from a deficiency of light." All these workers used the soybean. Mason and Maskell (1928) measured the carbohydrate

content of cotton leaves and concluded—"We infer an import of sugar by darkened (mature) leaves over a period of 24 hours . . . the correlation coefficients are, however, too small to make the inference certain . . . They establish a rather strong presumption that sugar was actually imported into the darkened leaves." In a more direct experiment with sugar-beet (Leonard 1939), plants were darkened for 5 days then transferred to sunlight with some leaves masked. He concluded "If there was any reversal of translocation from the illuminated to the darkened leaves, it was not measurable".

While, therefore, it is generally agreed that mature leaves import little or no sugar, even when darkened, we have now shown that there is no bar to such import. It may be demonstrated even in illuminated leaves or leaflets if the specific activity of the radiocarbon source is raised (expt. III-4). Its extent is usually increased if the leaf is darkened, or if other sinks are removed. Acropetal movement within the source leaf (attached or excised) may also be demonstrated under appropriate conditions, and marked import to necrotic areas suggested (expt. III-6) that high metabolism within a leaf could convert an area of mature leaf tissue into an efficient sink for assimilate. The darkening of healthy leaves lowers the concentration of mobile assimilates (expt. III-1, III-2), but it may be that a high utilization of sugar is necessary before import becomes massive. On the other hand, the relative lack of import into mature leaves may well be due in part (as envisaged by Leonard and others—see Crafts 1951) to an active secretion of sugar into the phloem from the border parenchyma. It is hoped to use radiocarbon to test the possibility that such a secretion is not operative until the leaf is reaching its full size.

V. Acknowledgments

Financial assistance from Monsanto Chemicals (Australia) Limited and from the Commonwealth Bank (Rural Credits Account) is gratefully acknowledged. We also owe thanks to Dr. R. T. Leslie and Mr. G. W. Rogerson, Statistics Department, University of Melbourne, for Appendix I and for advice on statistics, and to the late Professor E. J. Maskell for his valuable criticisms of a draft of the manuscript.

VI. References

Aronoff, S. (1955).—*Plant Physiol.* **30**: 184–5.

Belikov, I. F. (1955).—*Fiziol. Rast.* **4**: 345–57.

Belikov, I. F. (1958*a*).—*Akad. Nauk. S.S.S.R. Doklady* **119**: 1236–39.

Belikov, I. F. (1958*b*).—*Akad. Nauk. S.S.S.R. Doklady* **120**: 904–6.

Crafts, A. S. (1951).—*Bot. Rev.* **17**: 203–84.

Crafts, A. S. (1956).—*Hilgardia* **26**: 287–334.

Goodall, D. W. (1946).—*Ann. Bot., Lond.* (N.S.) **10**: 305–38.

Leonard, O. A. (1939).—*Plant Physiol.* **14**: 55–74.

Mason, T. G., and Maskell, E. J. (1928).—*Ann. Bot., Lond.* **42**: 571–636.

Nelson, C. D., and Gorham, P. R. (1957*a*).—*Canad. J. Bot.* **35**: 339–47.

Nelson, C. D., and Gorham, P. R. (1957*b*).—*Canad. J. Bot.* **35**: 703–13.

Vernon, L. P., and Aronoff, S. (1952).—*Arch. Biochem. Biophys.* **36**: 383–98.

Thaine, R., and Walters, M. C. (1955).—*Aust. J. Biol. Sci.* **8**: 354–68.

EXPLANATION OF PLATES 1–3

PLATE 1

Experiment I-1: application chamber circular in cross section. Note overexposure at site of application of $^{14}CO_2$ (Figs. 2 and 3)

Fig. 1.—Radioautographs of stems: (*a*) source leaflet lateral; (*b*) source leaflet terminal.

Fig. 2.—Radioautographs of terminal source leaflet, petiole, apex, and expanding trifoliate leaves. Radioautographs not obtained with lateral leaflets of source leaf or with other mature leaves.

Fig. 3.—Radioautographs of lateral source leaflet, petiole, apex, and expanding trifoliate leaves. Radioautographs not obtained with other lateral or terminal leaflet of source leaf or with other mature leaves.

Fig. 4.—Radioautograph of root system—source leaflet terminal.

Fig. 5.—Radioautograph of root system—source leaflet lateral.

PLATE 2

Figs. 1 and 2.—Radioautographs of segments of soybean leaves: experiment III-1 (Fig. 1), experiment III-2 (Fig. 2). After destarching, each attached leaf was illuminated and $^{14}CO_2$ applied to the central (bright) area. All leaves were then completely masked. They were cut into segments, dried, and radioautographed after the periods (hr) stated. In Figure 2 each segment is approximately the size of the black rectangle.

Fig. 3.—Experiment II-2: radioautographs of source leaflets. Plants *A* and *C*, apical buds illuminated; plants *B* and *D*, apical buds masked. The apical bud of plant *B* was "plated", but did not record a radioautograph.

Fig. 4.—Experiment III-4: radioautographs of source leaflet (*s*). The results show import into mature primary leaves (*p*, *p*), lateral leaflets of source leaf (*l*, *l*), mature trifoliate leaf (*t*). Note that import into the apex (*a*) is much greater than that into the leaves.

PLATE 3

Fig. 1.—Experiment III-6, plant A: radioautograph of source leaflet (of which the upper half was masked), the two other leaflets of source leaf, and one of the (stalked) primary leaves. Outlines drawn on negative.

Fig. 2.—Experiment III-6, plant B: as in Plate 3, Figure 1, but the source leaflet shows movement of tagged assimilate into the masked upper half and accumulation around chlorotic spots. Both primary leaves are shown to be free of activity, but there is local activity in the lateral leaflets of the source leaf (see text).

Fig. 3.—Experiment III-7: radioautograph of leaf which was completely masked except at the application site, and cut into three segments before air drying. There has been little acropetal movement and no movement across the main vein.

Fig. 4.—Experiment III-8: radioautograph of source leaflet with masked upper half, stem apex removed, showing acropetal movement into masked half, little movement across the main vein.

APPENDIX I

By R. T. LESLIE* and G. W. ROGERSON*

STATISTICAL ANALYSIS OF RESULTS FROM EXPERIMENT II-8

Plants from experiment II-8 (see Table 4) were compared in a balanced, incomplete block design, two treatments per block, three blocks per replication. In addition, the experiment was replicated three times.

Two apical treatments—apex masked and apex illuminated—were introduced as split plots within the main design. The apical treatment was repeated on duplicate plants within each split plot.

There were thus three independent estimates of variability:

(1) Variation between duplicates, for four degrees of freedom in each block, giving a total of 36 degrees of freedom;

(2) Within-block variation, after allowing for treatment effects, for 15 degrees of freedom;

(3) Between blocks, for 7 degrees of freedom.

Strictly, (1) is appropriate for evaluation of apical treatments, (2) and (3) for leaf treatments, but as differences between (1), (2), and (3) were non-significant, a pooled error variance was used.

The usual analysis for balanced incomplete blocks was applied separately to the two apical treatment series, and subsequently comparisons were made between apical treatments.

As the bulk of the uncontrolled variation was thought to be due to variability between different plants rather than to the random nature of a radioactive count, a logarithmic transformation was applied, corrected zero counts being given an arbitrary value of 0·5.

(*a*) *Numerical Results*

The observed errors were as follows:

Type of Error	Degrees of Freedom	Mean Square
Inter-block	7	0·114083
Within-blocks	15	0·119150
Between duplicates	36	0·291590

These were not significantly different and were pooled to give an error of 0·225569 for 58 degrees of freedom. This was used as error in all tests of significance. A complete analysis of variance is given in Table 6.

(*b*) *Conclusions*

(1) The effect of masking the apex was not significant.

(2) The overall differences between levels of factor (L) were highly significant (at 1 per cent. level). The mean square for (L_m) was not significant, but that for (L_o) was highly significant (at 1 per cent. level). Cutting off the side

---

*Statistics Department, University of Melbourne.

leaves increased the amount of tagged assimilate reaching the apex by about 60 per cent.

(3) Differences between "similar" blocks were significant (at the 5 per cent. level). Thus, some reduction in the error mean square was obtained by carrying out the work in blocks. In view of this, the design adopted in the present experiment seems very suitable for this type of work.

At first sight it seems that conclusion (2) is at variance with the means quoted in Table 4 for the three groups L_i, L_m, L_o. It must be noted, however, that the three-leaf treatments are paired in different blocks. Blocks 1, 2, and 3 are very light in yield; blocks 4, 5, and 6 very heavy; and blocks 7, 8, and 9 quite heavy, and a rough indication of adjusted comparisons is obtained if one doubles the results in blocks 1, 2, 3. The effect is clearly to raise the means for groups L_i and L_o, leaving that for group L_m unchanged, and consequently the difference between groups L_i and L_m is reduced while that between groups L_m and L_o is increased. Proper calculation shows that the means 5·7, 14·0, and 16·6 are changed to —4·4, —0·9, and 5·4 (general mean zero), and the conclusion stated above becomes reasonable. The effect is more apparent still when the data have been transformed by taking logarithms, in order to make an analysis of variance valid. Finally, the two orthogonal comparisons between the three-leaf treatments considered most appropriate were $L_i - L_m$, $L_i + L_m - 2L_o$. These correspond to the direct evaluation of masking, and the comparison of plants with and without removal of leaves.

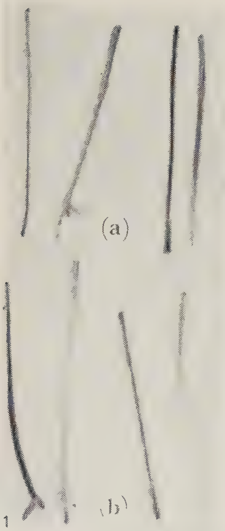
TABLE 6

COMPLETE ANALYSIS OF VARIANCE OF RESULTS FROM EXPERIMENT II-8

Source of Variation	D.F.	S.S.	M.S.
Leaf treatments			
Masking side leaves	1	0·435380	0·435380
Cutting off side leaves	1	4·958816	4·958816**
Blocks			
Varietal component	2	2·292764	1·146382**
Remainder	6	3·571101	0·595184*
Inter-block error	7	0·798583	0·114083
Apical treatments			
Masking effect	1	0·311524	0·311524
Masking effect × leaf treatments (eliminating blocks)	2	0·068601	0·034300
Masking effect × blocks			
Varietal component	2	0·458610	0·229305
Remainder	6	0·950654	0·158442
Inter-block × masking effect			
Error	7	0·377982	0·053997
Within blocks duplicate error	36	10·497288	0·291590
Total	71	24·721244	
Pooled error	58	13·083057	0·225569

$^{*}P < 0·05$. $^{**}P < 0·01$.

TRANSLOCATION IN THE SOYBEAN



1

2

3

4

5

TRANSLOCATION IN THE SOYBEAN

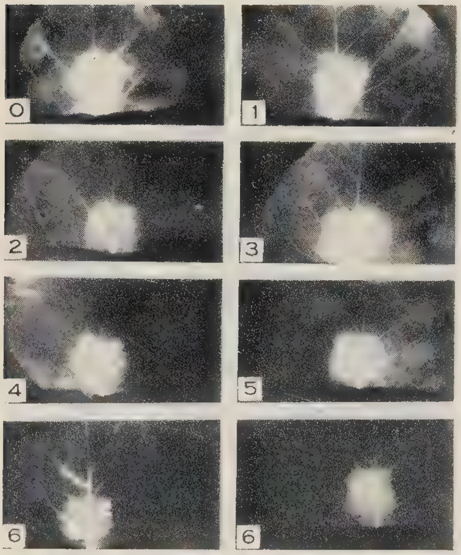


Fig. 1

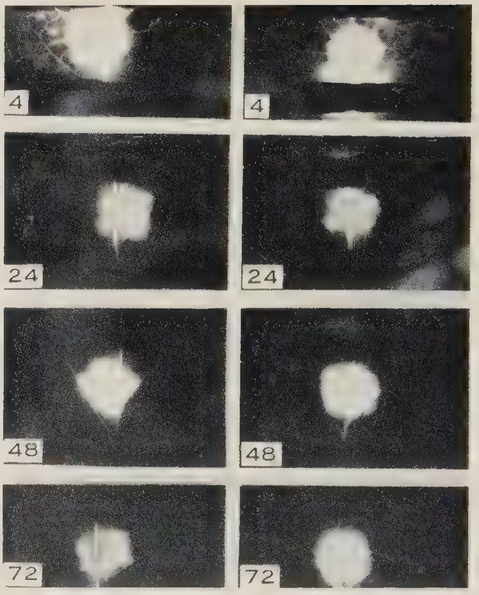


Fig. 2

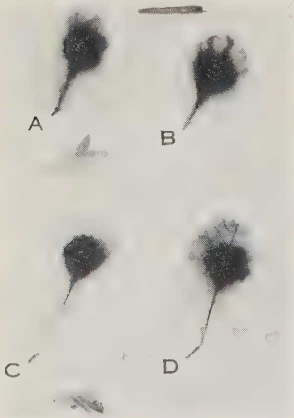


Fig. 3

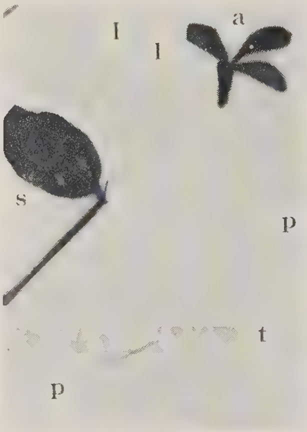
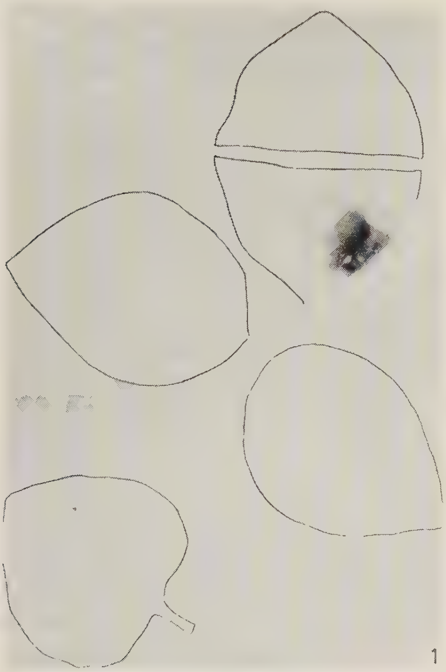
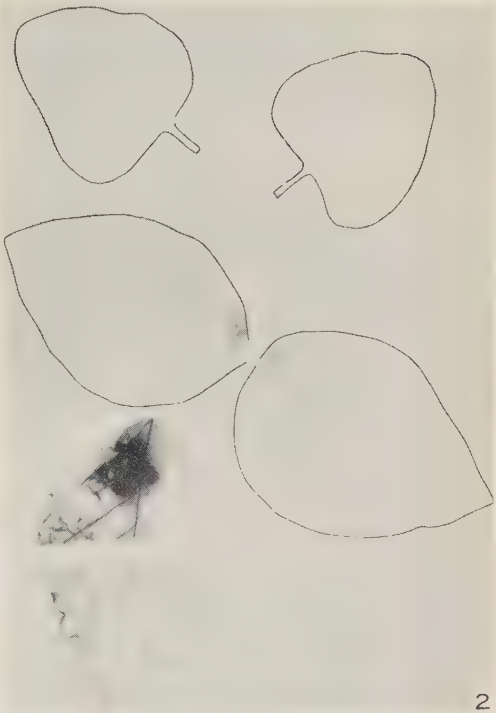


Fig. 4

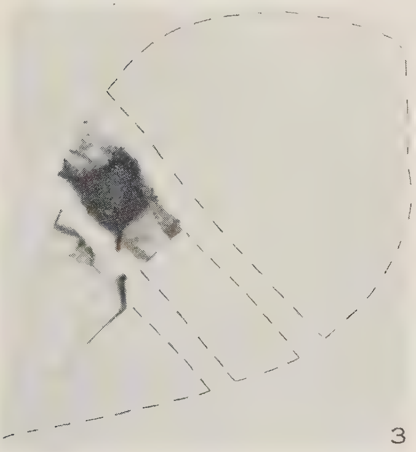
TRANSLOCATION IN THE SOYBEAN



1



2



3

4

RESIDUAL EFFECTS OF AUXIN, CHELATING AGENTS, AND METABOLIC INHIBITORS IN CELL EXTENSION

By D. J. CARR* and E. K. NG†

[*Manuscript received July* 10, 1959]

Summary

The growth-promoting action of auxin on wheat coleoptile segments can be separated from the act of cellular expansion by use of the technique of Cleland and Bonner (1956). The effects of a pretreatment with auxin ("residual effects") in hypertonic mannitol are manifested later when the coleoptiles are transferred to water. Auxin action, but not water uptake, requires aerobic conditions.

The residual effects of disodium ethylenediaminetetra-acetic acid (EDTA) resemble those due to 3-indolylacetic acid (IAA) in the following respects:

(1) Necessity for aerobic conditions during the pretreatment phase.

(2) Disappearance of residual effects when the coleoptiles are held in hypertonic mannitol for 150 min.

(3) Disappearance of residual effects in presence of an antiauxin (2,4,6-trichlorophenoxyacetic acid).

These facts are held to be contrary to what one might expect if the growth-promoting action of EDTA were attributable to chelation of calcium from the cell wall or other non-metabolic modes of action.

The residual effects due to EDTA, but not those due to IAA, are suppressed by 2,4-dinitrophenol (DNP) at concentrations of 2 mg/l or less. This is taken to mean that the mode of action of EDTA is different from that of IAA and involves certain pathways of oxidative metabolism which may be particularly sensitive to DNP.

As far as they have been investigated the residual effects due to *S*-carboxymethyldimethyldithiocarbamate (DTC) resemble those due to IAA both quantitatively and in their interaction with DNP.

In short-term experiments, DNP (a metabolic inhibitor) in very low concentrations may act synergistically with IAA, increasing the growth response to it; like iodoacetate, DNP can also induce auxin-like responses when acting alone. The concentrations which are effective are considerably below those normally used in experiments on the metabolic effects of these inhibitors. In long-term experiments, DNP has an auxin-like action only in the absence of added IAA.

It is suggested that auxin action, whether due to IAA, DTC, chelating agents, or metabolic inhibitors, is due to an induced shift in the metabolic balance of the cell in favour of syntheses and hence of growth. A suitable concentration of any of these substances may promote growth by affecting one of a number of different reactions. Important in this respect are certain steps in metabolism ("pacemaker reactions") at which "branching" occurs and substrates or phosphate esters may be competed for by different metabolic reactions.

*Botany School, University of Melbourne.
†Botany Department, Bedford College, London University.

I. Introduction

Cleland and Bonner (1956) have shown that the process of cell elongation in *Avena* coleoptiles can be separated into two main part processes. The first is dependent on the presence of oxygen and is promoted by auxin. Its main result is believed to be the plasticization of the cell wall. The second process may take place under anaerobic conditions and consists essentially of the uptake of water resulting in cellular expansion. This process is suppressed by hypertonic solutions. The "auxin action" phase can thus be experimentally separated from the "cell expansion" phase. First, the auxin is allowed to act for some time on the tissue which is aerated but prevented from expanding by a suitably high external osmotic concentration. Then the tissue is allowed to expand in water under conditions in which the action of auxin is blocked by a suitable inhibitor, e.g. lack of oxygen. The response of the tissue to auxin in the aerobic phase is expressed as cell expansion in the final anaerobic period. Cell expansion, i.e. water uptake, is very rapid and is not affected by the application of auxin. It is possible with this technique to test, in the final period, the "residual effects" brought about by auxin, effects which are independent of the act of cellular expansion.

The principles underlying this technique were first applied by Heyn (1931) and by Thimann (1951). The method as developed by Cleland and Bonner has been used by Cooil and Bonner (1957) in studying the interaction of calcium and potassium on cell wall plasticity. We have used it to compare the modes of action of chelating agents, which have been shown to have auxin-like activity (see Carr and Ng 1959), with that of auxin.

II. Materials and Methods

Segments 10 mm long of coleoptiles of wheat (cv. Gabo) were prepared in the manner previously described (Carr and Ng 1959). Lots of 15 or 20 segments were used for each test solution. Each lot of segments was subjected to three successive periods of treatment. During the first, or pretreatment, period, the segments were immersed for 45 min in 20 ml of 0·3M mannitol buffered with 0·015M K_2HPO_4–citric acid buffer containing a known concentration of IAA, EDTA, or DTC.* A control without IAA or chelating agent was also set up. In the second, or transition, period the segments were transferred for 30 min to a solution containing 0·3M mannitol (to prevent expansion) and an inhibitor of auxin action. This treatment ensures that the action of auxin is completely blocked before the segments are allowed to expand. During the third, or expansion period, the segments were immersed in 20 ml of buffered glass-distilled water and allowed to expand in the presence of an inhibitor of auxin action for 90 min, after which time there is no further expansion. Then the segments were removed and measured.

Solutions were made up in glass-distilled water. In the experiments the solutions were contained in glass vials and the segments were held in stainless steel wire baskets below the surface of the solutions to prevent the possibility of contact

---

*The following abbreviations will be used throughout: IAA, β-indolylacetic acid; EDTA, disodium ethylenediaminetetra-acetic acid; DTC, *S*-carboxymethyldimethyldithiocarbamate; DNP, 2,4-dinitrophenol; 2,4,6-T, 2,4,6-trichlorophenoxyacetic acid.

with air at the surface. During the pretreatment period air was bubbled through the solutions. During the second and third periods anaerobic conditions were maintained (as the inhibitor of auxin action) by bubbling nitrogen through the solutions. In transferring the baskets from one solution to another they were plunged rapidly into the new solution which previously had been saturated with nitrogen. In this manner no more than a few seconds of exposure to air was allowed during the transfers between periods of treatment. Nitrogen was purified by first passing it through two solutions of aged alkaline pyrogallol to absorb oxygen, and then through two scrubbers containing water. Where necessary the gas was passed through a solution of KOH before entering the pyrogallol solution. The bubbling of the test solutions in the transition and expansion periods was commenced at least 40 min before the actual beginning of the transition period. To ensure that the solutions remained saturated with nitrogen a flow of nitrogen was maintained until the end of the experiment.

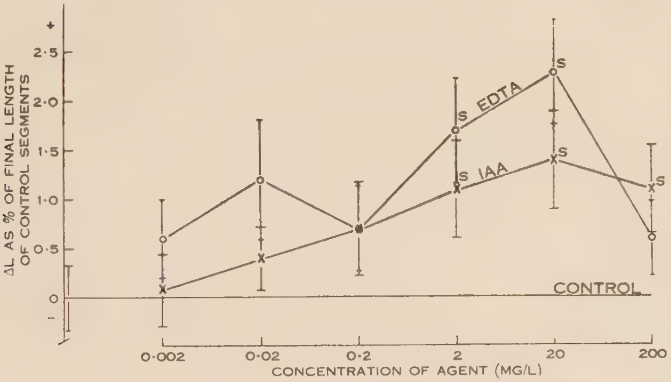


Fig. 1.—Residual effects of EDTA and IAA on extension growth of wheat coleoptile segments. Ordinates: differences (ΔL) between mean lengths of IAA- and EDTA-treated segments after expansion expressed as a percentage of the final lengths of the control segments. Each point is the mean of three experiments, 15–20 segments per treatment. Pretreatment period: aerobic plus mannitol; transition period: anaerobic (nitrogen) plus mannitol; expansion period: anaerobic plus water. All solutions buffered to pH 4·9 with citric acid–phosphate buffer. In Figures 1–10, a vertical line through each point denotes the standard error; points marked *S* differ significantly at the 5 per cent. level from the controls, which had neither IAA nor EDTA present during the pretreatment period.

The results are expressed as the difference (ΔL) between the average lengths of the treated and non-treated segments after expansion. Significance was estimated at the 5 per cent. probability level, using the *t*-test.

III. Experiments

(*a*) *Residual Effects of IAA and EDTA*

The solutions were buffered to pH 4·9. Figure 1 is drawn from the means of three experiments (not those given in Table 1). It is seen that IAA gave a residual effect at 2, 20, and 200 mg/l and EDTA at 2 and 20 mg/l. The differences between the residual effects due to these substances are not significant and although EDTA gave slightly greater effects than IAA the curves run fairly close together. In one experiment (503, Table 1) both IAA and EDTA gave residual effects at all concentrations. In this experiment, in which the pH was 4·4, the residual effects were much greater than those reported by Cleland and Bonner (1956) for oat coleoptiles and at the highest concentration EDTA produced a very large effect. Initially it was believed that this might have been due to the use on this occasion of an unusually pure sample of nitrogen. On other occasions (e.g. 514, Table 1) "industrial nitrogen" or mixtures of nitrogen and 5 per cent. carbon dioxide (scrubbed as indicated above) were used. The possibility was considered that (despite assurances by the manufacturers to the contrary) traces of carbon monoxide might occur in all but the "pure" nitrogen. Experiment 515 (Table 1) was performed to check this. The gas was scrubbed with a carbon monoxide absorber but the responses were not as great as those obtained in the exceptional experiment. It is possible, but not likely, that the differences in pH between experiment 503 and other experiments in this series might account for the difference in responses, but the exceptional result of experiment 503 could not in any way be repeated.

TABLE 1

DIFFERENCE (ΔL) BETWEEN AVERAGE LENGTHS OF TREATED AND UNTREATED (CONTROL) SEGMENTS AFTER EXPANSION

Values in italics differ from the controls (treatment with distilled water, buffered to the stated pH) at the 5 per cent. level

Expt.* No.	Agent	pH	ΔL (mm)					
			Concentration of Agent (mg/l):					
			0·002	0·02	0·2	2	20	200
503	IAA	4·4	*0·30*	*0·23*	*0·31*	—	*0·36*	*0·11*
	EDTA	4·4	*0·46*	*0·36*	*0·46*	—	*0·70*	*1·45*
514	IAA	4·9	0·07	0·07	0·02	0·11	*0·30*	*0·22*
	EDTA	4·9	0·06	*0·24*	0·03	*0·20*	*0·16*	*0·11*
515	IAA	4·9	0·08	0·03	*0·13*	*0·12*	0·07	*0·10*

*Expt. 503, pure nitrogen used. Expt. 514, scrubbed industrial nitrogen used. Expt. 515, industrial nitrogen passed through ammoniacal cuprous chloride and then scrubbed before use.

(*b*) *Residual Effects of IAA and DTC*

At pH 4·4 (Fig. 2) IAA produced significant residual effects at almost all concentrations. With DTC significant residual effects were also obtained at most concentrations and the residual effect of DTC at 20 mg/l was greater than that of

IAA at the same concentration. At pH 7·4 (Fig. 3) and 7·6 residual effects were also obtained, but here a lower concentration was optimal, as was expected from the effects of pH on activity of DTC already described (Ng and Carr 1959). However, the large residual effect of DTC at 0·2 mg/l and pH 7·4 is not significantly different from that due to IAA at the same concentration.

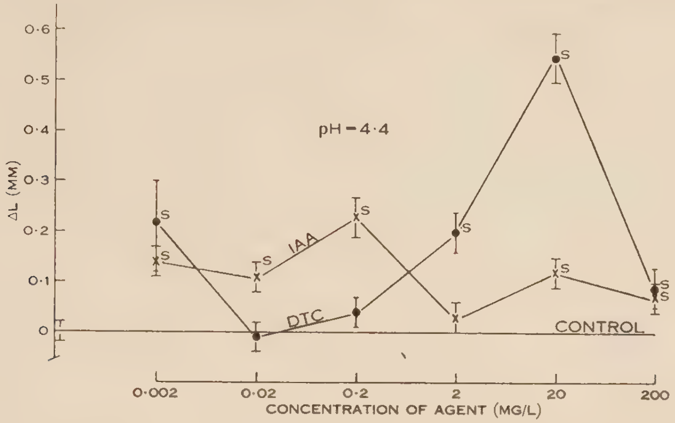


Fig. 2.—Residual effects of DTC and IAA on extension growth of wheat coleoptile segments; pH 4·4. Ordinates: differences (ΔL mm) between treated and control segments after expansion. Each point represents the mean difference for 15–20 segments.

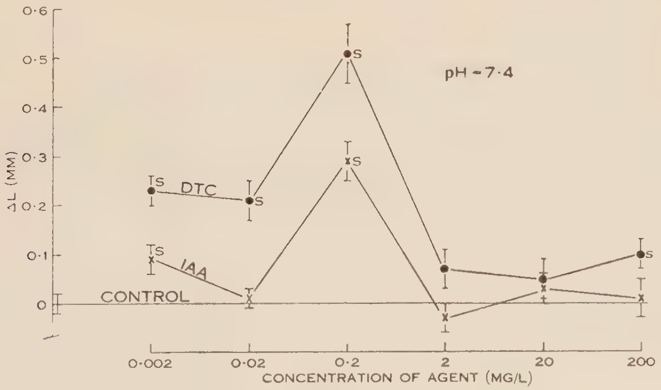


Fig. 3.—Residual effects of DTC and IAA on extension growth of wheat coleoptile segments; pH 7·4. Ordinates: differences (ΔL mm) between treated and control segments after expansion. Each point represents the mean difference for 15–20 segments.

(c) *Aerobic Conditions during the Pretreatment Period Necessary for Residual Effects of IAA and EDTA*

The demonstration of residual effects due to EDTA similar to those due to IAA opens up the possibility of experiments to find out whether they are brought about in the same way. For instance, auxin will produce residual effects only if the pretreatment phase is aerobic. The results of an experiment in which IAA and EDTA were allowed to act on coleoptile segments in the usual way, but with nitrogen instead of air during the pretreatment period, are shown in Figure 4. The pH was 5·1.

Residual effects were obtained with neither IAA nor EDTA. In fact, some of the segments were slightly shorter at the end of the experiment than at the beginning. From the data of Cleland and Bonner (1956) it is evident that oat coleoptile segments also shrink following a similar treatment. Whether the greater shrinkage following EDTA treatment has any biological meaning is doubtful. In any case it seems clear that, like IAA, EDTA must act in the presence of oxygen in order to produce residual effects.

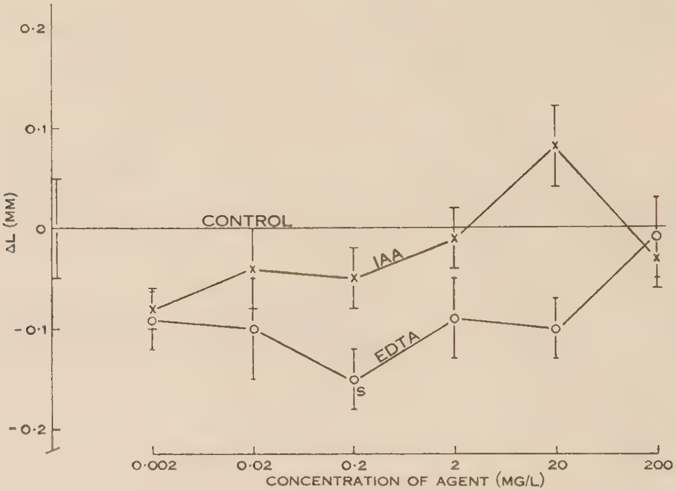


Fig. 4.—Residual effects due to IAA and EDTA applied during an *anaerobic* pretreatment period; pH 5·1. The graphs illustrate the necessity for aerobic conditions during the pretreatment period in order to produce residual effects.

(*d*) *Suppression of Residual Effects by Prolongation of the Transition Period*

Prolongation of the transition period appears to suppress the residual effect of IAA (Cleland and Bonner, loc. cit.). Results of experiments to investigate the effect of prolonging the transition period from the normal 30 min to 150 min are shown in Table 2. No significant residual effects were obtained, either with IAA or with EDTA, and in very many treatments, particularly with EDTA, the segments shrank.

(*e*) *Inhibition Due to the Presence of an Antiauxin* (2,4,6-*T*) *during Transition and Expansion Periods*

According to Cleland and Bonner (1956) the antiauxin 2,4,6-trichlorophenoxyacetic acid exerts a "strictly competitive inhibition of auxin-induced growth in *Avena* coleoptile sections." Residual effects due to IAA could be partially obliterated in the presence of 2,4,6-T during the transition and expansion periods. We find that 2,4,6-T at 2×10^{-4}M can completely obliterate the residual effects due to IAA acting on wheat coleoptile segments in solutions buffered to pH 4·9. The antiauxin was added during the transition and expansion phases. In the presence of this substance no significant residual effects were obtained with any concentration of either IAA or EDTA. Thus the residual effects due to EDTA resemble those due to IAA in their reversibility by 2,4,6-T.

Table 2

EFFECT OF PROLONGING THE TRANSITION PERIOD FROM 30 TO 150 MIN ON EXPANSION OF SEGMENTS

Values are differences (ΔL) between average lengths of treated and untreated (control) segments after expansion. Negative values of ΔL indicate that the segments shrank

Agent	pH	ΔL (mm) Concentration of Agent (mg/l):					
		0·002	0·02	0·2	2	20	200
IAA	4·9	−0·05	−0·20	−0·01	−0·34	−0·34	−0·33
EDTA	4·9	−0·25	−0·09	−0·07	−0·08	−0·38	−0·32
IAA	5·0	0·02	0·01	0·09	0·05	0·16	0·13
EDTA	5·0	−0·04	−0·04	0·17	0·00	0·01	−0·08

(*f*) *Effects Due to DNP during the Transition and Expansion Periods*

In seeking differences in the mechanism by which EDTA and IAA bring about the residual effects described above, DNP was used as the inhibitor of auxin action in the transition and expansion phases instead of nitrogen. The concentration used was 2 mg/l, which Cleland and Bonner (1956) have shown not to prevent the appearance of residual effects due to IAA. The results of experiments with IAA and EDTA at pH 4·5 and 5·5, and with IAA and DTC at a pH of 7·6 are summarized in Figures 5 and 6.

The results confirm the statement of Cleland and Bonner that 2 mg/l of DNP does not suppress the residual effects due to IAA, since all but the lowest concentration of IAA gave significant residual effects. On the other hand, EDTA either produced no effects at all or small effects with no proportionality to concentration. The clear and obvious difference in behaviour of the residual effects due to IAA and EDTA in the presence of DNP indicates that the mode of action of EDTA on the growth of the coleoptile must differ in some essential manner from that of auxin. At pH 7·6 IAA again gave large residual effects but the relationship to IAA concentration was somewhat erratic, 2 mg/l producing particularly large responses (Fig. 6). DTC gave residual effects at the higher concentrations rising to a maximum at the highest concentration, and the responses were almost as great as those induced by IAA at equivalent concentrations.

(g) *Action of very low Concentrations of DNP on Residual Effects of IAA and EDTA*

Although DNP at 2 mg/l discriminates against the residual effects of EDTA in cell extension, it might be argued that this would not occur with lower concentrations of DNP. To test this a range of low concentrations of DNP was applied during the transition and expansion periods, while air was bubbled through the solutions. Only one concentration of IAA or of EDTA was used (2 mg/l) and the solutions were buffered to pH 4·5. The results, shown in Figure 7, were surprising in that even with very low concentrations of DNP there were no residual effects of EDTA. On the other hand, IAA produced quite large residual effects in the presence of DNP at 2 mg/l or less but not at 5 mg/l. Indeed, as can be seen from Figure 7, the residual effect of IAA in the presence of 1 mg/l DNP was greater than that induced by IAA alone with no DNP added during the transition or expansion periods. Two conclusions may therefore be drawn from the data. Firstly, support is given to the view that the mechanism of action of EDTA is radically different from that of

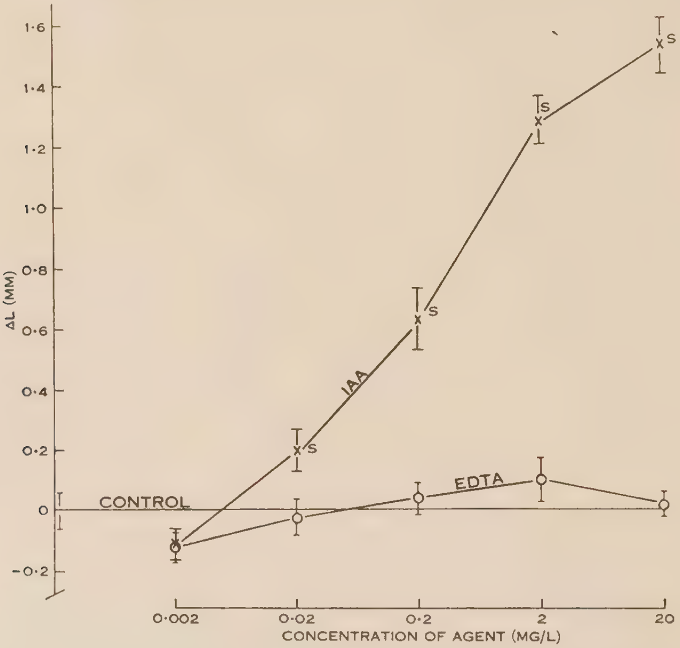


Fig. 5.—Residual effects due to IAA and EDTA with the inhibitor DNP (2 mg/l) applied instead of nitrogen during the transition and expansion periods; pH 4·5. Note the virtual elimination of residual effects due to EDTA but not those due to IAA.

IAA. Secondly, a metabolic inhibitor, DNP, in very low concentration appears to act synergistically with IAA, increasing the growth response to it.

(*h*) *Residual Effects of DNP and Iodoacetic Acid*

If DNP can enhance the response to IAA then it may be able to stimulate growth in the absence of added auxin, and to produce residual effects. In testing this, different concentrations of DNP were used in the aerobic pretreatment period and nitrogen was bubbled through the solutions (buffered to pH 5·0) during the remaining periods. DNP was present only during the pretreatment period. As may be seen from the results (Fig. 8), DNP either had no effect or was slightly inhibitory at the higher concentrations (0·1 mg/l and above) but at 0·001 and 0·01 mg/l it actually caused a stimulation of growth.

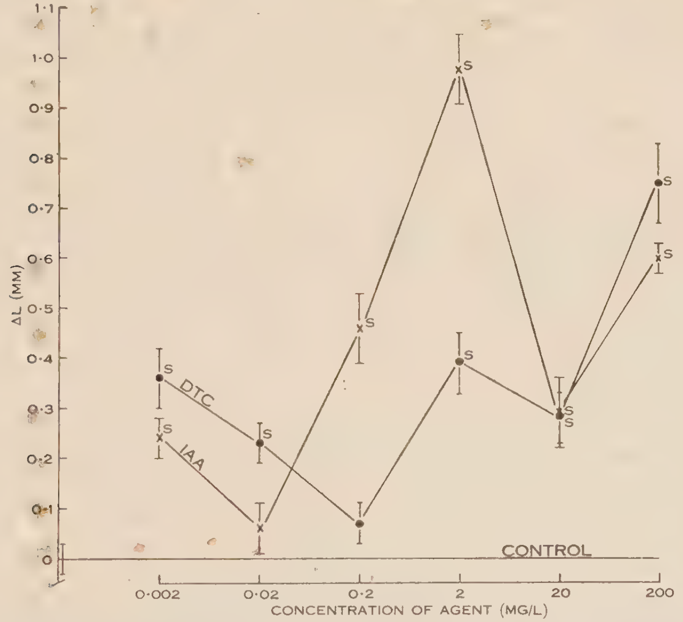


Fig. 6.—Residual effects due to IAA and DTC with the inhibitor DNP (2 mg/l) applied instead of nitrogen during the transition and expansion periods; pH 7·6.

With previous reports (e.g. Slocum and Little 1957) of growth stimulation by low concentrations of another metabolic inhibitor, iodoacetate, in mind we have sought to determine whether iodoacetate would also induce residual effects. The method was the same as that described for DNP, except that the solutions were buffered to pH 4·8. The results (Fig. 9) indicate that, like DNP, iodoacetate can also produce residual effects in the absence of an exogenous supply of auxin. The stimulatory range appears to be small and the optimum around 0·002 mg/l. Neither this nor the low concentrations of DNP which can induce residual effects on growth are generally considered to be effective in the inhibition of metabolic processes.

(i) *Effects of Low Concentrations of DNP in the Normal Straight-growth Test with Wheat Coleoptiles*

In view of the surprising result described above some low concentrations of DNP were tested in the normal straight-growth test. The technique used was that already described (Ng and Carr 1959) with the exception that the incubation time was 22 instead of 24 hr. The solutions were buffered to pH 5·0. The results are shown in Figure 10. When auxin (0·2 mg/l) was added to the solutions, all concentrations of DNP were inhibitory. In the absence of added auxin, however, the lowest concentrations of DNP, with an optimum at 0·01 mg/l were found to promote growth.

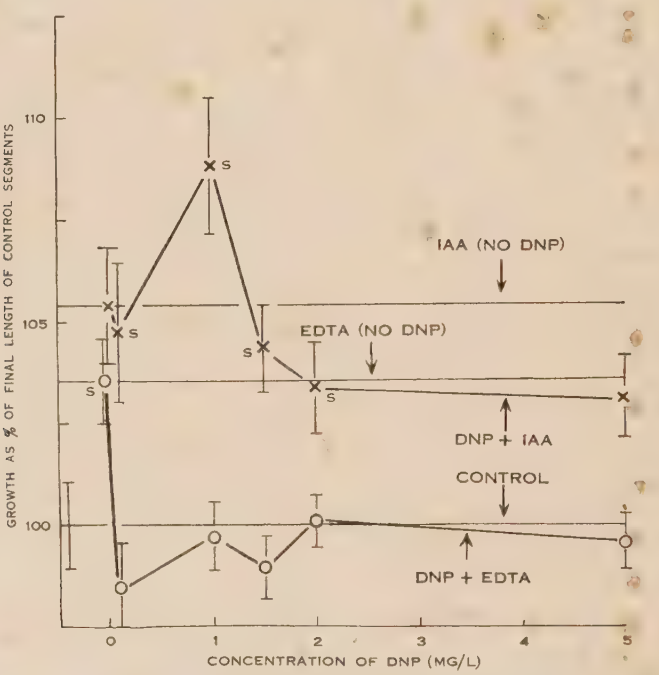


Fig. 7.—Residual effects due to IAA and EDTA (both at 2 mg/l) at pH 4·5 with DNP at different concentrations applied during the transition and expansion periods. Upper graph, IAA applied during the pretreatment period, aerobic conditions during transition and expansion periods. Significance (*S*) is with respect to the control (lower graph) with neither IAA nor EDTA in pretreatment period. Note (1) synergism of DNP (at 1 mg/l) with IAA at 2 mg/l; (2) complete blockage of residual effects due to EDTA at all concentrations of DNP.

IV. Discussion

The data corroborate the findings of Cleland and Bonner (1956) concerning the residual effects due to IAA but also shed light on the nature of growth-promotion by chelating agents such as EDTA. Although the residual effects due to EDTA are very similar in many respects to those induced by IAA (requirement for aerobic conditions in the pretreatment period, obliteration by 2,4,6-T, or by prolonged immersion of the segments in hypertonic mannitol) it does not follow that the modes of action of these substances are identical. In fact, the mode of action of EDTA is differentiated from that of IAA by its response to the presence of DNP. In previous papers (Carr and Ng 1959; Ng and Carr 1959) we have examined the effects of chelating agents on coleoptile growth in the light of the hypothesis (Heath and Clark 1956*a*, 1956*b*) that these substances sequester calcium from the cell wall, thus increasing its plasticity. It is abundantly clear from the data given above that this hypothesis cannot explain the mode of action of EDTA on growth. It cannot explain, for instance, the dependence of the residual effects due to EDTA on oxidative metabolism. The possibility that calcium may be involved in the "stiffening" of the cell wall during a prolonged transition period has been raised by the work of Cooil and Bonner (1957) but on any hypothesis that EDTA chelates calcium from the cell wall the residual effect of EDTA should persist over a prolonged transition period.

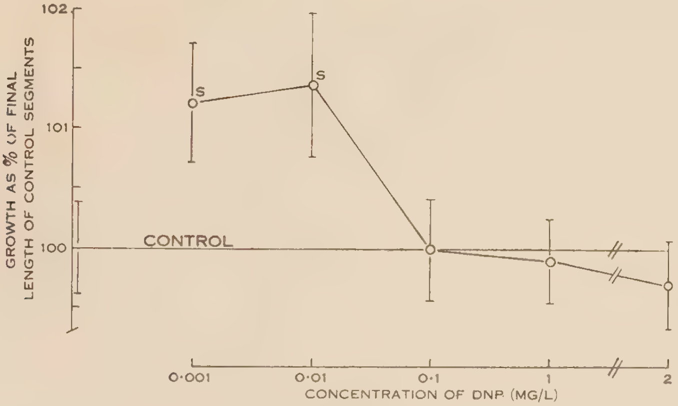


Fig. 8.—Residual effects due to pretreatment with DNP; pH 5. Nitrogen applied during transition and expansion periods.

On the other hand, it is difficult to explain why the antiauxin 2,4,6-T should be able to reverse the plasticization of the cell wall caused either by IAA or by EDTA, even under anaerobic conditions (in which neither IAA nor EDTA is effective). No explanation of the mode of action of this antiauxin has been put forward by Cleland and Bonner (1956). It is difficult to imagine that it merely stimulates demethylation of pectins or the formation of calcium pectate because it is effective even when present only during the expansion phase. The fact that suitable concentrations of DNP in the transition and expansion periods can obliterate "residual" effects which have already been completed or at least initiated in the pretreatment period may mean that the persistence of the residual effects during the transition period must depend on persistence of the train of metabolic events initiated by the growth-promoting substance.

Unlike that of IAA, the action of EDTA appears to depend on phases of metabolism which are very sensitive to DNP. It should be stressed, however, that this conclusion applies only to the short-term experiments. In longer experiments, lasting 22 hr or more, DNP at 2 mg/l, a concentration which is ineffective in short-term experiments, is inhibitory to growth. At least two explanations of this are possible. In Stenlid's (1949) experiments the carrot leaves treated with 10^{-4}M DNP at pH 5 took up more oxygen than the controls over the first hour, but after that time oxygen uptake was strongly inhibited. On the other hand, Thimann and Bonner (1948) have shown that coleoptiles become, with age, more sensitive to inhibition by iodoacetate. Some such aging phenomenon may be responsible for inhibition, in experiments of long duration, by DNP concentrations which are not inhibitory in the residual-effect experiments. Thimann and Bonner also confirmed the statement of Commoner and Thimann (1942) that, in the presence of sucrose and IAA, iodoacetate promotes the growth of oat coleoptiles. According to Slocum and Little (1957) iodoacetate and arsenite stimulate growth by partially inhibiting the utilization of pyruvate or α-ketoglutarate in energy production, thus releasing more hexose carbon to the synthesis of structurally essential compounds. This redistribution is dependent on the auxin concentration because "at the optimal concentration of auxin we have an ideal dynamic balance at which the division of substrate carbon between the two processes of energy production and utilization for structure is precisely optimal". Some such scheme as that proposed by Slocum and Little may explain the stimulatory effects of DNP on growth, if, in low concentration, this compound enables a new dynamic equilibrium in the utilization of hexose to be set up in the growing cell. It may be supposed that with 20 mg/l of IAA in the medium and other conditions "normal" the metabolic balance of wheat coleoptile segments is optimal for growth. If DNP is added at, say 10^{-7}M (a concentration which has no, or only stimulatory, effects on oxygen uptake according to Simon (1953) and French and Beevers (1953)) the greater utilization of hexose in energy production may inhibit growth (as shown in Fig. 10) but stimulate respiration. In

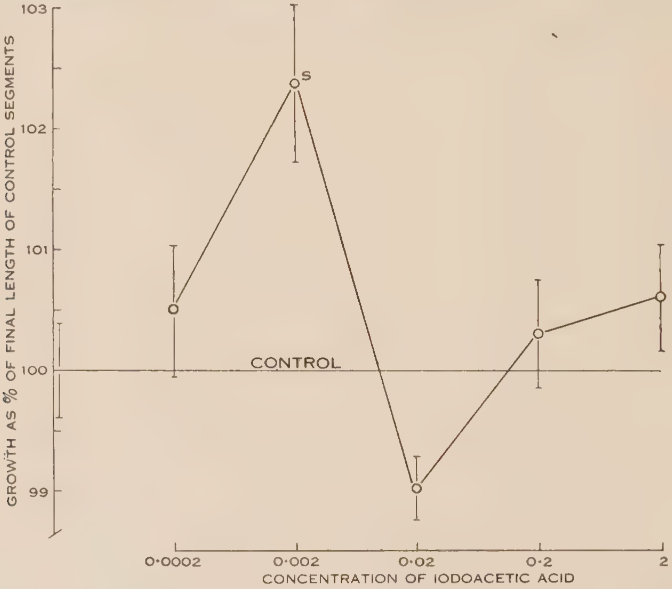


Fig. 9.—Residual effects due to iodoacetic acid; pH 4·8. Nitrogen applied during transition and expansion periods.

the absence of added auxin, however, the same concentration of DNP might shift the balance of metabolism in favour of growth. The more or less complete uncoupling of oxidative phosphorylation resulting from comparatively large concentrations of DNP would be quite unfavourable for growth, since synthetic reactions could not then proceed. The level of auxin necessary so that stimulation by DNP may be displayed must be rather low, and is probably about the normal endogenous level in coleoptile segments. This may well be the explanation of the difference in response of different kinds of tissues of *Parthenocissus* and sunflower to "concentrations of DNP which are not known to affect respiratory processes or the accumulation of ATP" reported by Klein (1957). Habituated and callus tissues showed stimulation of growth by concentrations between 10^{-10} and 10^{-7}M, but crown gall tissues, which are known to be rich in growth hormones, showed only inhibition by DNP at all concentrations between 10^{-11} and 10^{-6}M. Klein has offered no explanation of his results, but they appear to agree remarkably well with those described above for coleoptile segments.

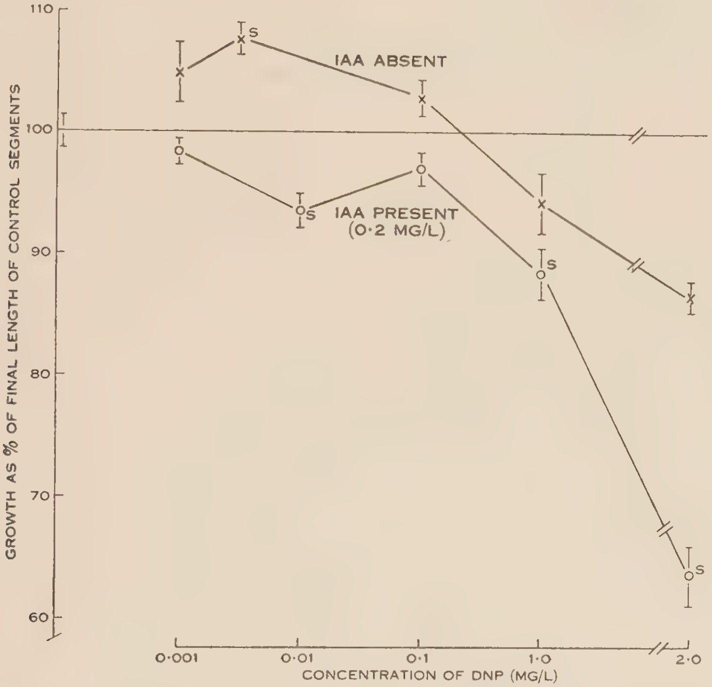


Fig. 10.—Effects of low concentrations of DNP on wheat coleoptile segments incubated for 22 hr, pH 5, in the presence and absence of IAA. In the presence of IAA all concentrations of DNP are inhibitory. In the absence of exogenous IAA, growth is stimulated by DNP at 0·01 mg/l.

Opinions differ on the ability of DTC to act as a complexing or chelating agent. Wain (private communication) believes that it has little or no such activity. The general opinion, however, is that it elicits true auxin responses (see Ng and Carr 1959) and this is supported by the data presented in this paper. The fact that DTC gives large residual effects which, like those due to IAA, are insensitive to DNP at 2 mg/l.

strengthens the impression that its mode of action is closely similar to that of IAA. It is possible that DTC and IAA may act on one or more of the "pacemaker" reactions (Krebs 1957), the stages of cell metabolism most "liable to be influenced by inhibitors or hormones" thereby bringing about a subtly balanced utilization of carbohydrate which is favourable to growth processes. Chelating agents may also affect pacemaker reactions and thus, indirectly, act as growth-promoting substances. Probably they intervene at the electron-transfer stage where they have been shown to have effects on respiration (James 1953).

Since both respiratory and synthetic processes in the cell are often dependent on the level of phosphate esters it is possible that the effects of growth-promoting substances are due partly to effects on the level of phosphate esters. For instance, although the role of ATPase in the cell is still uncertain, DNP, EDTA, and Ca^{++} are all known to have marked effects on this enzyme in mitochondrial preparations (Forti 1957). Stimulation and inhibition, by different concentrations of thyroxine, of syntheses dependent on ATP in animal cells have been explained by Hoch and Lipmann (1953) in terms of changes in the rate of turnover of phosphate acceptors. Too tight or too loose a linkage between respiration and phosphorylation is "undesirable" and optimal concentrations of thyroxine maintain a "favourable" balance. In view of the rather common phenomenon of an "optimum concentration" which many substances display (e.g. IAA, DNP) when they both stimulate and inhibit a specific process, it may be suggested that they act in metabolism in a way similar to that envisaged by Slocum and Little (1957) whose scheme is a specific realization of the proposals of Hoch and Lipmann and of Thimann (1956).

As Audus (1954) has remarked, the trend in auxin physiology has been to seek a "master reaction" controlled by auxin. There may in fact be no single "master reaction". A suitable concentration of any metabolite or poison or other effective agent whether auxin, chelating agent, metabolic inhibitor, or substrate, which is able to shift the balance of metabolism of the cell (which must at the outset be potentially capable of growing), in favour of synthesis of protein or of wall components will be growth-promoting. The number of ways in which this might be achieved could be large, although certain critical "pacemaker" reactions may be particularly susceptible.

V. Acknowledgments

We are indebted to Mr. J. M. Robinson, I.C.I.A.N.Z., Merrindale Research Station, Victoria, for a gift of 2,4,6-T.

These studies were made in part fulfilment of the requirements for the degree of M.Sc. in the University of Melbourne by the junior author (E. K. Ng).

VI. References

Audus, L. J. (1954).—The mechanism of auxin action. Rapports et communications. 8th Int. Congr. Bot., Paris. p. 138.

Carr, D. J., and Ng, E. K. (1959).—The sequestration of calcium from preparations of wheat coleoptile cell walls. *Physiol. Plant.* **12**: 264.

Cleland, R., and Bonner, J. (1956).—The residual effect of auxin on the cell wall. *Plant Physiol.* **31**: 350.

COMMONER, B., and THIMANN, K. V. (1942).—On the relation between growth and respiration in the *Avena* coleoptile. *J. Gen. Physiol.* **24**: 49.

COOIL, B. J., and BONNER, J. (1957).—The nature of growth inhibition by calcium in the *Avena* coleoptile. *Planta* **48**: 696.

FORTI, G. (1957).—Adenosinetriphosphatase activity of pea mitochondria. *Physiol. Plant.* **10**: 899.

FRENCH, R. C., and BEEVERS, H. (1953).—Respiratory and growth responses induced by growth regulators and allied compounds. *Amer. J. Bot.* **40**: 660.

HEATH, O. V. S., and CLARK, J. E. (1956*a*).—Chelating agents as plant-growth substances—possible clue to the mode of action of auxin. *Nature* **177**: 1118.

HEATH, O. V. S., and CLARK, J. E. (1956*b*).—Chelating agents as growth substances. *Nature* **178**: 600.

HEYN, A. J. (1931).—Der Mechanismus der Zellstreckung. *Rec. Trav. Bot. Neérl.* **28**: 113.

HEYN, A. J. (1940).—The physiology of cell elongation. *Bot. Rev.* **6**: 515.

HOCH, F. L., and LIPMANN, F. (1953).—The uncoupling of respiration and phosphorylation by thyroid hormones. *Proc. Nat. Acad. Sci., Wash.* **40**: 909.

JAMES, W. O. (1953).—The use of respiratory inhibitors. *Annu. Rev. Pl. Physiol.* **4**: 59.

KLEIN, R. M. (1957).—Growth and differentiation of plant tissue cultures. In "Rhythmic and Synthetic Processes in Growth". (Ed. Dorothea Rudnick.) (Princeton Univ. Press.)

KREBS, H. A. (1957).—Control of metabolic processes. *Endeavour* **16**: 125.

NG, E. K., and CARR, D. J. (1959).—Effects of pH on the activity of chelating agents and auxins in cell extension. *Physiol. Plant.* **12**: 275.

SIMON, E. W. (1953).—Mechanisms of dinitrophenol toxicity. *Biol. Rev.* **28**: 453.

SLOCUM, D. H., and LITTLE, J. E. (1957).—Growth stimulation of *Avena* coleoptile. *Plant Physiol.* **32**: 192.

STENLID, G. (1949).—Some notes on the effect of sodium azide, 2,4-dinitrophenol and ortho-phenanthroline upon oxygen consumption in green leaves. *Physiol. Plant.* **2**: 61.

THIMANN, K. V. (1951).—Studies on the physiology of cell enlargement. Tenth symposium on development and growth. *Growth* **15** (suppl.): 5.

THIMANN, K. V. (1956).—Promotion and inhibition: twin themes of physiology. *Amer. Nat.* **90**: 145.

THIMANN, K. V., and BONNER, W. D. JR. (1948).—Experiments on the growth and inhibition of isolated plant parts. I. The action of iodoacetate and organic acids on the *Avena* coleoptile. *Amer. J. Bot.* **35**: 271.

POTENTIATION OF LATERAL ROOT INDUCTION BY ROOT INITIALS IN ISOLATED FLAX ROOTS

By P. L. Goldacre*

[*Manuscript received April* 24, 1959]

Summary

When β-indolylacetic acid (IAA) (10^{-5}M) is added to isolated flax roots, lateral roots are induced to form adventitiously. If some lateral root initials are already present when IAA is added, newly induced primordia form preferentially immediately adjacent to them. It is suggested that a chemical stimulus, a kinin, originating from the existing root meristems interacts with IAA to induce further cell division in the pericycle. It is proposed that kinin formation may be a normal accompaniment of cell division.

I. Introduction

The production of a substance by the stem apex, and its transport to the stem, where it may induce adventitious root formation, is one of the earliest-known examples of physiological correlation (Went and Thimann 1937). This root-forming substance was identified as auxin (Thimann and Koepfli 1935).

It has been recognized that other endogenous metabolites may at times limit the induction of root initials by auxins. The rhizocaline hypothesis (Went 1938; Bouillenne 1950) proposes that although auxin is a prerequisite for the regenerative growth involved in the rooting process, it is a second material, rhizocaline, originating from stems, leaves, or cotyledons which specifically evokes root formation. Cooper (1936) and Van Overbeek and Gregory (1945) showed that in addition to auxin, some other substance is transported from the leaves and is necessary for adventitious root formation on stem cuttings. The cotyledons of the pea seed (Rippel 1937; Torrey 1950; Fries 1954) or the older portion of the isolated pea root (Peckett 1957) appear to contribute some substance which promotes lateral root formation. This factor can be diluted out by serial subculture of the main axis meristem (Torrey 1950). In other cases, known growth factors such as thiamine (Went, Bonner, and Warner 1938), nicotinamide and tryptophan (Galston 1948, 1949), biotin (Went and Thimann 1937), adenine (Torrey 1956), vitamins K and H (Hemberg 1953), and arginine together with tryptophan and histidine (Goldacre and Unt, unpublished data 1955) have been shown to enhance the formation or development of lateral roots.

The first observable act in the initiation of lateral roots is the occurrence of adventitious cell divisions in the tissue giving rise to the primordia, usually the pericycle in herbaceous plants (Van Tiegham and Douliot 1888; Crooks 1933). It is becoming increasingly clear that, for cell division to occur in many situations, it is necessary to have present both an auxin and a member of the class of compounds

---

* Division of Plant Industry, C.S.I.R.O., Canberra.

known as kinins (Steward and Shantz 1956; Braun 1957; Skoog and Miller 1957). It seems not unreasonable to suppose that root meristems may contain or produce kinins, and that the induction of lateral root primordia may require the participation of a kinin. This paper describes an observation which suggests that root meristems do contain a diffusible substance which, in the presence of auxin, promotes the formation of further root initials.

II. Materials and Methods

Seeds of flax (*Linum usitatissimum* L. 'Ventnor') were surface-sterilized by wetting with alcohol and immersing for 15 min in sodium hypochlorite (final concentration equivalent to 0·075N chlorine). The alkaline solution was washed off with 1 per cent. saturated bromine water. Seeds were spread onto sterile petri plates containing basal medium plus 0·7 per cent. agar and incubated at 25°C.

After 4 days, terminal 10-mm sections were excised from the radicles, transferred to 40 ml sterile medium contained in 150-ml conical flasks, and incubated in darkness at 25°C.

Basal medium contained Bonner's macronutrient salts (Bonner 1940), White's micronutrient salts (White 1943), with the addition of 0·05 mg/l $CuSO_4.5H_2O$, 0·13 mg/l $(NH_4)_6Mo_7O_{24}.4H_2O$, 0·1 mg/l thiamine, 0·5 mg/l nicotinic acid, 0·1 mg/l pyridoxine, and 2 per cent. sucrose. The pH was adjusted to 5·5 with $KHCO_3$.

After 3 days incubation, when the average length of the main axis was about 7 cm, there were present 8–12 unemerged lateral primordia ranging in length from 0·02 to 0·10 mm (Plate 1, Fig. 1). At this stage 0·4 ml sterile 10^{-3}M β-indolylacetic acid (IAA) was added (final concn. equals 10^{-5}M) and the roots were further incubated and sampled at time intervals.

Roots were fixed by vacuum infiltration with 2·4 per cent. formaldehyde in 70 per cent. aqueous ethanol. After 30 min they were washed in 70 per cent. ethanol, then cleared by immersion overnight in Sartory's clearing agent (33 ml glycerol, 17 ml lactic acid, 20 g phenol, and 20 ml water). Unemerged root initials were then photographed, or their positions measured along the main axis using a dissecting microscope and ocular micrometer.

Other roots, after fixation, were embedded in wax, sectioned at 10 μ, stained with ruthenium red, mounted, and photographed.

III. Results

The effect of adding IAA at this concentration (10^{-5}M) is to inhibit the growth of the main axis meristem and to promote the formation of lateral root primordia. When some primordia are already present at the time of adding the IAA, the new primordia which form consequently are not randomly distributed, but arise predominantly in the immediate vicinity of pre-existing root initials (Plate 1, Figs. 2 and 3). This results in clumps of root initials appearing at irregular intervals. The distribution of induced primordia in a typical root 20 days after adding IAA is shown diagrammatically in Figure 1.

In Figure 2 a measure is given of the relative frequencies of occurrence of new primordia near to and distant from the pre-existing laterals for four typical roots. Since the intervals between pre-existing laterals vary, they were each subdivided into the central half and two terminal quarters. Counts of primordia in the central half were subtracted from the pooled counts for the two terminal quarters for each interval (see Fig. 2, *inset*). This difference was then plotted against the interval length. First it is seen (Fig. 2) that almost all differences are positive, i.e. there are more laterals in the two terminal quarters than in the central half of each interval. Further, the longer the interval, the greater is the difference. This would be expected if some influence originating from the pre-existing laterals were propagated a limited distance during the experimental period. The few cases in which the difference approximates zero or is negative are for very short or very long intervals. In the first case, induced primordia may occupy the whole interval; the original lateral, being thicker, occupies a significant portion of the space counted as terminal quarters: this leads to a negative difference in this form of representation. In the second case, where the interval is large, a large number of randomly disposed initials are induced along the central half.

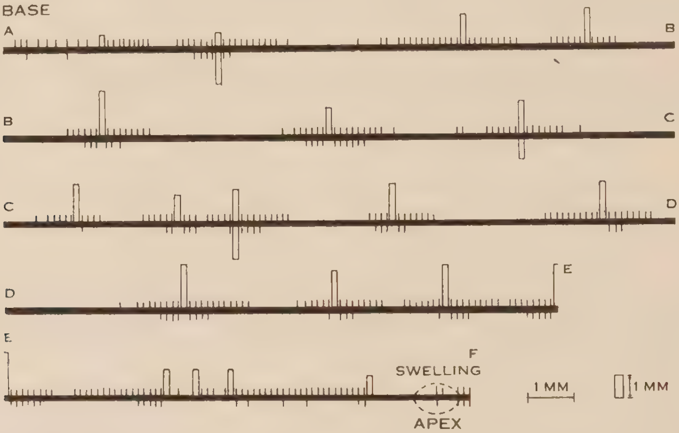


Fig. 1.—Distribution of induced lateral primordia on one typical root. IAA (10^{-5}M) added after 3 days growth; root fixed after a further 20 days incubation. For the sake of representation the main axis AF is here subdivided into AB, BC, CD, DE, EF. Open blocks represent pre-existing laterals, and their lengths represent the length at the time of fixing.

Subsequent development of the induced primordia is inhibited by the presence of the IAA, but if the IAA is removed, the laterals grow out, giving a tufted appearance (Plate 1, Fig. 4).

Sampling at time intervals after adding IAA reveals that a wave of cell-division activity begins in the pericycle adjacent to the pre-existing primordia (Plate 1, Fig. 5; Plate 2, Fig. 1) and in the radial plane containing the protoxylem and moves along the root in both directions from these points (Plate 2, Fig. 2).

The tissue produced as a result of this cellular proliferation tends to segment and form organized structures which eventually develop into lateral root primordia

(Plate 1, Fig. 3; Plate 2, Fig. 3). The margins of such proliferating regions appear, in turn, to stimulate *neighbouring* (quiescent) pericycle parenchyma cells into dividing (Plate 2, Figs. 2 and 4). Thus the influence of the original meristem spreads consecutively in both directions, stimulating cellular proliferation which, upon further development, gives rise to many primordial root apices packed close to each other, and with centres approximately 0·13 mm apart (Plate 1, Fig. 3; Plate 2, Fig. 3). The old cortical tissue tends to disintegrate in these regions.

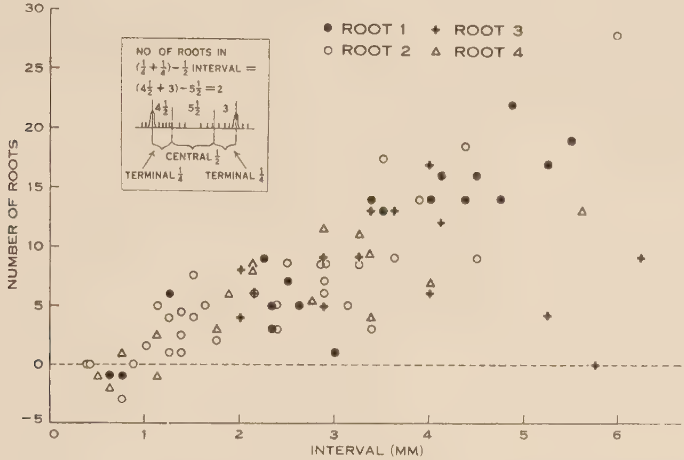


Fig. 2.—Distribution of induced lateral primodia on four typical roots. Each interval between pre-existing laterals has been divided into the central half and the two terminal quarters. For each interval the number of induced root initials in the central half has been subtracted from the sum of the initials in the two terminal quarters. This difference has been plotted against the interval length. Note that the differences are almost always positive; and that the longer the interval, the greater is the difference.

In cases where those lateral roots present at the time of adding IAA have already emerged, i.e. when the lateral root apex is separated from the stele of the main axis by an elongation zone, the influence of such roots in potentiating primordium formation at their bases is lost. This suggests that the stimulus arises in the meristematic zone itself and does not pass through the elongation zone.

IV. Discussion

These results indicate that some stimulus originating from the immediate neighbourhood of unemerged root initials potentiates the induction of new root initials by IAA. Time studies suggest that the stimulus is in some way propagated short distances in both directions along the root. The stimulus acts only upon the pericycle tissue lying in the same plane (i.e. in the radial plane containing the protoxylem), usually on the same side of the root, and to a lesser extent on the opposite side of this diarch root (Plate 2, Fig. 3).

This localization of response and indeed the almost total restriction of the normal formation of lateral primordia to the pericycle immediately adjacent to the protoxylem (Van Tiegham and Douliot 1888; Crooks 1933; observations this paper) suggest that the protoxylem itself may play a part in the induction process.

It has been concluded by a number of authors (Rippel 1937; Geissbühler 1953; Fries 1954; Torrey 1956; Peckett 1957) that root branching is inhibited by the presence of the main axis meristem of peas, and extracts from such meristems have been shown to be inhibitory to the initiation of lateral root meristems (Libbert 1956; Torrey 1956). Contrarily to this antagonistic behaviour, the young meristems described in the present work *promote* the formation of their own kind. This suggests that the stimulus produced by the pre-existing primordia cannot be merely a nutrient metabolite specifically required for cell division, for then the meristematic region would demand it, rather than produce it. The substance postulated here has the property of being produced by dividing cells, and of stimulating cell division in the presence of IAA; i.e. given favourable conditions it can catalyse its own formation. The chemical identity of the stimulus merits further investigation. We have been unable to obtain any stimulation of lateral root formation by kinetin or by *N*,*N′*-diphenyl urea.

This concept raises the important question of the of mechanism self-perpetuation of meristems (cf. Sinnot 1956). One interesting possibility is that kinin production may be a normal accompaniment of cell division. Any such kinin leaking from a lateral root primordium into neighbouring pericycle tissues would, in the presence of added auxin, be expected to induce cell division there. One would further suppose that in the developing root apex, the cell extension zone somehow inactivates or limits the movement of this kinin with the result that division is confined to the apex and the whole organ does not become engulfed in a wave of cell proliferation. This supposition is in accord with the observation that lateral roots do not, in the presence of IAA, potentiate the formation of new primordia adjacent to their bases once an elongation zone has been established.

V. REFERENCES

BONNER, J. (1940).—On the growth factor requirements of isolated roots. *Amer. J. Bot.* **27**: 692.

BOUILLENNE, R. (1950).—La rhizogenèse. *Année Biol.* (3) **26**: 597.

BRAUN, A. C. (1957).—A physiological study of the nature of autonomous growth in neoplastic plant cells. *Symp. Soc. Exp. Biol.* **11**: 133.

COOPER, W. C. (1936).—Transport of root-forming hormone in woody cuttings. *Plant Physiol.* **11**: 779.

CROOKS, D. (1933).—Histological and regenerative studies on the flax seedling. *Bot. Gaz.* **95**: 209.

FRIES, N. (1954).—Chemical factors controlling the growth of the decotylised pea seedling. *Symb. Bot. Upsaliens.* **13**: 1.

GALSTON, A. W. (1948).—On the physiology of root initiation in excised asparagus stem tips. *Amer. J. Bot.* **35**: 281.

GALSTON, A. W. (1949).—Indoleacetic acid–nicotinic acid interactions in the etiolated pea plant. *Plant Physiol.* **24**: 577.

GEISSBÜHLER, H. (1953).—Untersuchungen über die korrelative und hormonale Steuerung der Seitenwurzelbildung. *Ber. schweiz. bot. Ges.* **63**: 27.

HEMBERG, T. (1953).—The effect of vitamin K and vitamin H on root formation in cuttings of *Phaseolus vulgaris* L. *Physiol. Plant.* **6**: 17.

LIBBERT, E. (1956).—Untersuchungen über die Physiologie des Adventivwurzelbildung. II. Die korrelative Beeinflussung der Adventivwurzelbildung durch andere Organe, insbesondere durch die Wurzel. *Planta* **48**: 157.

PECKETT, R. C. (1957).—The initiation and development of lateral meristems in the pea root. I. The effect of young and mature tissue. *J. Exp. Bot.* **8**: 172.

RIPPEL, K. (1937).—Umkehr der Seitenwurzelgenese bei Leguminosen als korrelative Störung. *Ber. dtsch. bot. Ges.* **55**: 288.

SINNOT, E. W. (1956).—Botany and morphogenesis. *Amer. J. Bot.* **43**: 526.

SKOOG, F., and MILLER C. O. (1957).—Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp. Soc. Exp. Biol.* **11**: 118.

STEWARD, F. C., and SHANTZ, E. M. (1956).—In "The Chemistry and Mode of Action of Plant Growth Substances". (Ed. R. L. Wain and F. Wightman.) (Butterworths Scientific Publications: London.)

THIMANN, K. V., and KOEPFLI, J. B. (1935).—Identity of the growth-promoting and root-forming substances of plants. *Nature* **135**: 101.

TORREY, J. G. (1950).—The induction of lateral roots by indoleacetic acid and root decapitation. *Amer. J. Bot.* **37**: 257.

TORREY, J. G. (1956).—Chemical factors limiting lateral root formation in isolated pea roots. *Physiol. Plant.* **9**: 370.

VAN OVERBEEK, J., and GREGORY, L. E. (1945).—A physiological separation of two factors necessary for the formation of roots on cuttings. *Amer. J. Bot.* **32**: 336.

VAN TIEGHAM, P., and DOULIOT, H. (1888).—Recherches comparatives sur l'origine des membres endogènes dans les plantes vasculaires. *Ann. Sci. Nat. Bot.* (7) **8**: 1.

WENT, F. W. (1938).—Specific factors other than auxin affecting growth and root formation. *Plant Physiol.* **13**: 55.

WENT, F. W., BONNER, J., and WARNER, G. C. (1938).—Aneurin and the rooting of cuttings. *Science* **87**: 170.

WENT, F. W., and THIMANN, K. V. (1937).—"Phytohormones". (The MacMillan Publishing Co.: New York.)

WHITE, P. R. (1943).—"A Handbook of Plant Tissue Culture." p. 105. (Jacques Cattell Press: Lancaster, Pa.)

EXPLANATION OF PLATES 1 AND 2

PLATE 1

Fig. 1.—A cleared typical root after 3 days growth showing the development of lateral root initials before 10^{-5}M IAA was added. ×21.

Fig. 2.—Portion of a cleared root 10 days after 10^{-5}M IAA was added. ×2·4.

Fig. 3.—Enlarged portion of a cleared root 10 days after 10^{-5}M IAA was added. Note the extensive cellular proliferation in the pericycle and the beginnings of organization of root apices. ×60.

Fig. 4.—Resumed growth of induced primordia after removal of IAA by exposure to blue fluorescent light in the presence of 0·1 mg/l riboflavin. Note tufted appearance. ×0·95.

Fig. 5.—Median longitudinal section of root, 3 days after 10^{-5}M IAA was added. *P*, lateral root primordium present at time of adding IAA; *CD*, cellular division occurring in pericycle parenchyma immediately adjacent to the primordium. ×110.

PLATE 2

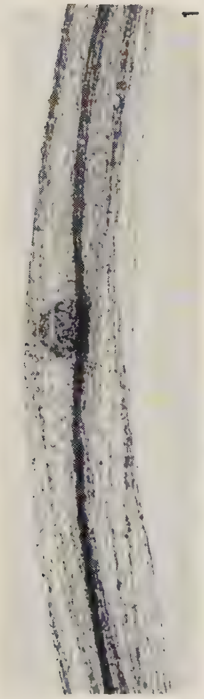
Fig. 1.—Enlargement of portion of Plate 1, Figure 5. ×350.

Fig. 2.—Longitudinal section of root 5 days after adding 10^{-5}M IAA. ×105.

Fig. 3.—Longitudinal section of root 17 days after adding 10^{-5}M IAA. ×30.

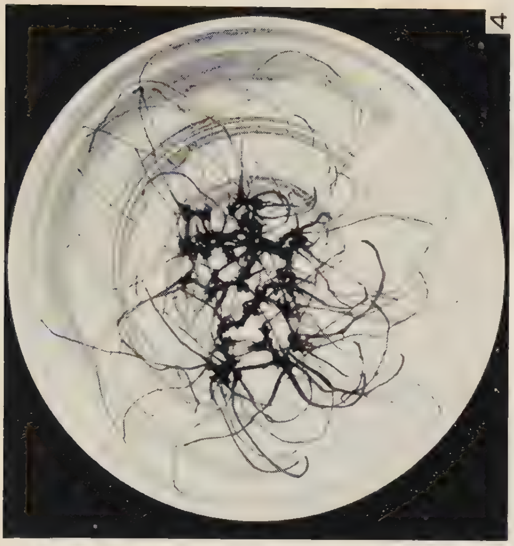
Fig. 4.—Longitudinal section of root 15 days after 10^{-5}M IAA was added, showing the front of the advancing wave of cell division in the pericycle parenchyma. *C*, cortex; *E*, endodermis; *PX*, protoxylem; *U*, original undivided pericycle parenchyma cells; *D*, pericycle parenchyma cells completing their first division; *P*, actively proliferating cells. The original lateral root is beyond the left of the picture. ×350.

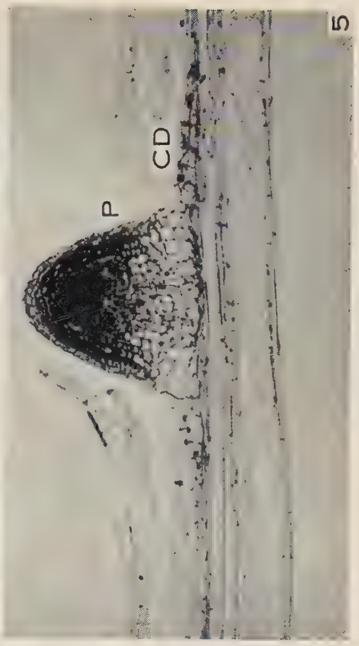
ROOT INDUCTION


1

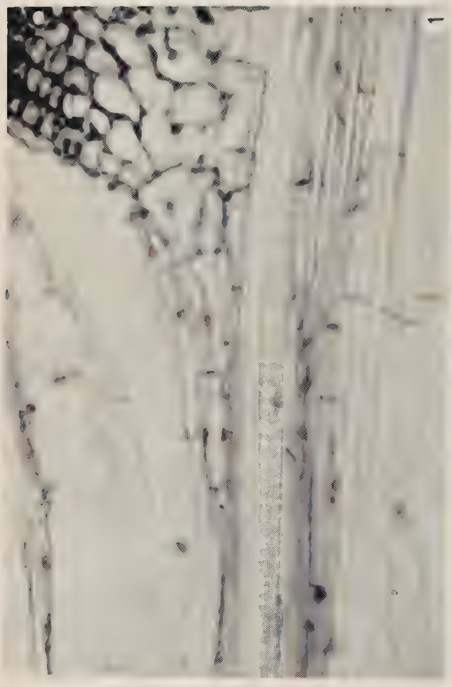
2

3

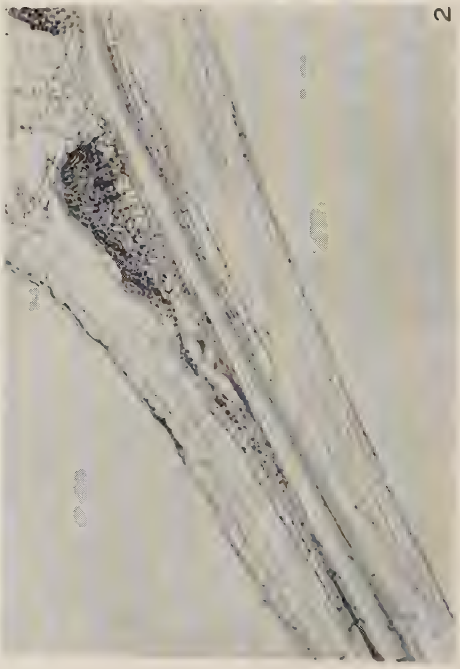

4


5

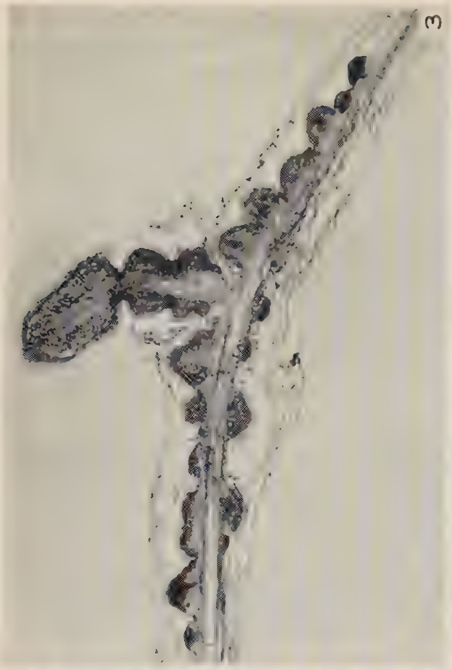
ROOT INDUCTION



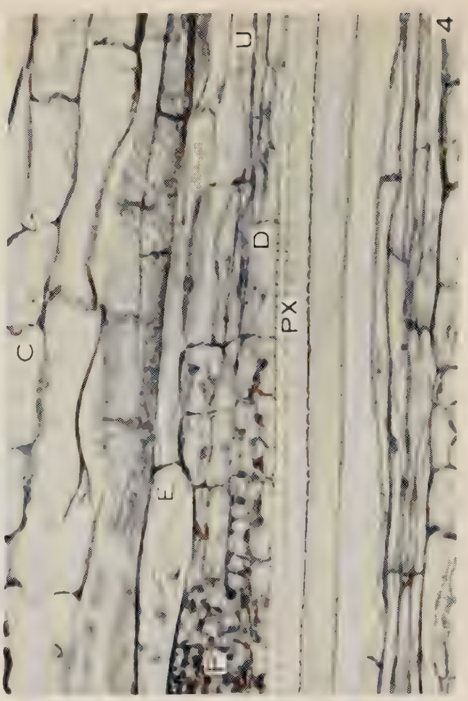
1



2



3



4

IONIC RELATIONS OF CELLS OF *CHARA AUSTRALIS*

I. ION EXCHANGE IN THE CELL WALL

By J. Dainty* and A. B. Hope†

[*Manuscript received April* 23, 1959]

Summary

Measurements of ion exchange were made between isolated cell walls of *Chara australis* and an external solution. Comparison between intact cells and cell walls showed that nearly all the easily exchangeable cations are located in the cell wall. The wall is shown to consist of "water free space" (W.F.S.) and "Donnan free space" (D.F.S.); the concentration of indiffusible anions in the D.F.S. is about 0·6 equiv/l. This finding is contrary to past suggestions that the D.F.S. is in the cytoplasm of plant cells.

The time course of cation exchange in the wall, measured with the aid of the radioactive isotopes ^{22}Na and ^{45}Ca, is shown to be complex. The cation exchange could be analysed into fractions with characteristic half-times ranging from about 100 sec for the "fast" fraction of sodium exchanging with ^{22}Na in the wall to many minutes for the "slower" fractions.

The fast fraction of both sodium and calcium exchange is shown to be diffusion-limited by a stationary film of the order of 100 μ thick outside the wall. It is suggested that the slowing of the remainder of the exchange is due to a combination of steric hindrance in micropores <100 Å in diameter in the wall, together with an electrostatic retardation in the electric double layers of the D.F.S.

I. Introduction

Recent studies of the ion-exchange processes in cells of *Nitellopsis* (MacRobbie and Dainty 1958) have revealed three processes separable on the basis of characteristic exchange half-time. These were (with respective half-times for K^+ exchange):

(1) Exchange with "free-space" ions ($\sim$1 sec).
(2) Exchange with "non-free-space" ions ($\sim$1 hr), said to be cytoplasmic.
(3) Exchange with vacuolar ions ($\sim 10^3$ hr).

The present study is an attempt to characterize in detail the free-space exchange in internodal cells of the alga *Chara australis* R. Br. var. *nobilis*.

Several papers have lately stressed the probability that the "Donnan free space" (D.F.S.) (Briggs, Hope, and Pitman 1958) is located in the cytoplasm with a rather larger "water free space" (W.F.S.) contributed by the cell-wall spaces and intercellular spaces (see Briggs 1957; Briggs and Robertson 1957). The possible contribution of the cell wall to the initial cation exchange, which is now widely regarded as adjustment of a Donnan system (Briggs, Hope, and Pitman 1958; Middleton and Russell 1958) has not been explored, although Sutcliffe (1957) states that the *cellulosic* components of the cell contributed very little to the initial ion exchange in his experiments with disks of red beet.

---

*Biophysics Department, University of Edinburgh.

†Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., and Botany School, University of Sydney.

Earlier experiments (Hope and Stevens 1952; Hope 1953), sometimes quoted as evidence for including the cell cytoplasm in the free space, have been rightly criticized (Briggs 1957) as being inadequate. Further, the considerations of Walker (1957), whose experiments with *Nitella* point to the existence of a plasmalemma at the surface of the cell which hinders ion exchange in the cytoplasm, suggest that the situation should be reconsidered. The present material was chosen because of the possibility that various components of the cell could be studied separately. This first part of the series discusses the exchangeable Na^+ and Ca^{2+}, the iodide free space (I.F.S.) and mannitol free space (M.F.S.) of the cell wall, and the kinetics of the movement of these ions and mannitol between the cell wall and external solution.

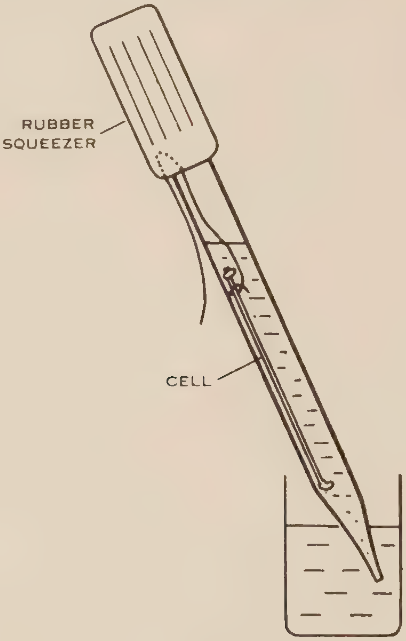


Fig. 1.—Method used to elute radioactivity from *Chara australis* cells and walls. A length of cotton was loosely tied around the cell and anchored between the rubber squeezer and glass tube.

II. Experimental Methods

(*a*) *Material*

Strands of *Chara australis* were collected from ponds and subcultured in a polythene-lined concrete tank containing tap water with a pond-mud base. Several weeks before they were needed for experiment individual strands were removed to a glass aquarium and placed in an artificial pond water (A.P.W.) of the following composition: NaCl, 1 m-equiv/l; $CaCl_2$, 0·5 m-equiv/l; KCl, 0·1 m-equiv/l. This corresponded roughly to the ion content of the field pond water.

Individual internodal cells together with the nodes, which contained a number of much smaller cells, were cut from a strand and placed in petri dishes in A.P.W. Cells in this condition survived many weeks with normal cytoplasmic streaming and appearance, and with the normal low rate of exchange of sap ions.

The cells were usually 4–7 cm long and 1–1·5 mm in diameter. The third, fourth, or fifth cell from the apex of the strand was chosen if possible.

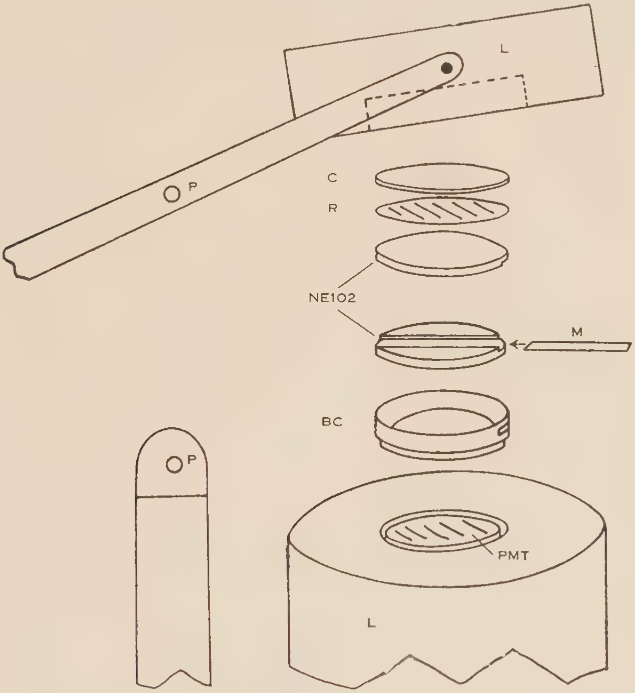


Fig. 2.—Expanded view of scintillation counter used to measure the radioactivity of 1 [^{14}C]-mannitol: *L*, lead castle, with lid pivoted at *P*; *PMT*, photomultiplier tube; *BC*, brass carrier for scintillating plastic; *NE*102, scintillating plastic slabs; *M*, mica slide carrying thin sample of mannitol; *R*, aluminium foil reflector; *C*, light-tight cap.

(*b*) *Experimental Procedures*

After periods of equilibration of up to 7 days in A.P.W., cells were placed in an A.P.W. of the same composition but labelled with the appropriate tracer ion, i.e. ^{22}Na^{+}, ^{45}Ca^{2+}, or ^{131}I^{-}. The specific activity was usually 0·1–10 μc/ml. In experiments with internodal cells the nodes were kept free of radioactivity by means of "Perspex" stocks, the nodal few millimetres being bathed in inactive solution, frequently changed. After a certain time the cell was lightly blotted and placed in a drawn-out glass pipette and a rubber squeezer placed over one end (see Fig. 1). The radioactivity was then eluted into aliquots (10 ml for liquid counting; 2 ml, subsequently dried, for solid counting) of inactive A.P.W. by continually sucking up liquid around the cell and expelling it. This operation took a minimum time of

1 sec and was repeated several times in a given time interval. By this method the liquid medium was kept stirred, and, because the volume of the aliquot was large compared with that of the cell or wall, the transfer of radioactivity was not limited by increase in the external specific activity. The amounts of activity in the aliquots after various times were determined using conventional Geiger tubes and scalers, or the scintillation counter described below.

In experiments using ^{131}I as a tracer to determine anion free space,* the liquid aliquots were made alkaline and some solid sodium thiosulphate added to prevent oxidation of the iodide and subsequent loss to the atmosphere.

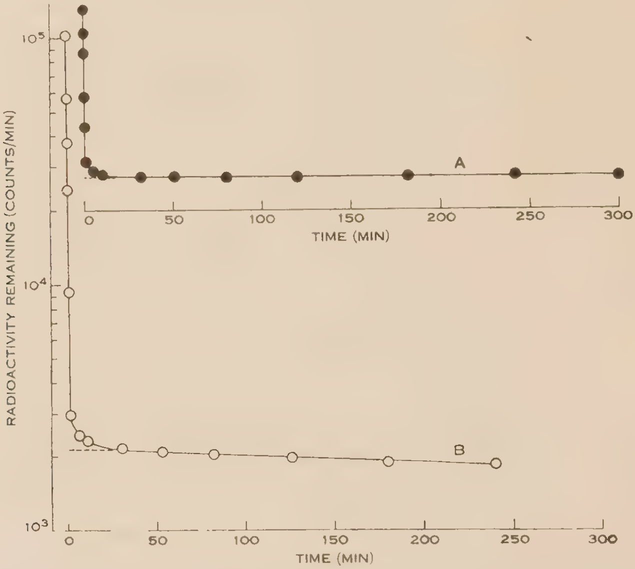


Fig. 3.—Radioactivity remaining in a *Chara australis* cell (*A*) and its isolated wall (*B*) plotted on a logarithmic scale against time of elution. Curves (*A*) and (*B*) have been separated by 10 min on the time axis to prevent overlapping.

Isolated cell walls were obtained by cutting the nodes from whole cells and scraping gently to remove the contents. Contamination by cytoplasmic interpenetration of the wall cannot be excluded. The time course of exchange in walls was measured in the same way as with the cells described above. When the total amount of an ion was to be measured, the activity of a cell wall was often counted directly after drying the wall on a planchette. The specific activity of the solution with which the wall had been in equilibrium was then determined by counting a sample of it, usually 20 μl, which was dried in the centre of the planchette together with a segment of inactive wall. Self-absorption and geometry complications were thus minimized.

---

*^{131}I was used instead of ^{36}Cl because the latter is available at such low specific activities that it could have been used only at very high external concentrations.

The M.F.S. of cells and walls was measured by equilibrating the free space with 5 mM mannitol solution which was made radioactive by adding 1 μc/ml of 1 [^{14}C]-mannitol. After careful blotting the activity was eluted into 2-ml aliquots of distilled water or A.P.W. The radioactivity was then determined in a scintillation counter of special design (Fig. 2), as follows: The liquid samples were dried on sheets of white mica 20 mm by 45 mm by 20 μ thick and placed between two slabs of scintillating plastic† each 3 mm thick and 50 mm in diameter. These were optically coupled to a type 6097 photomultiplier tube. A counting efficiency of approximately 22 per cent. for "infinitely thin" samples was obtained with the optimum setting of high tension voltage and discriminator bias, at which the background was usually 80 counts/min.

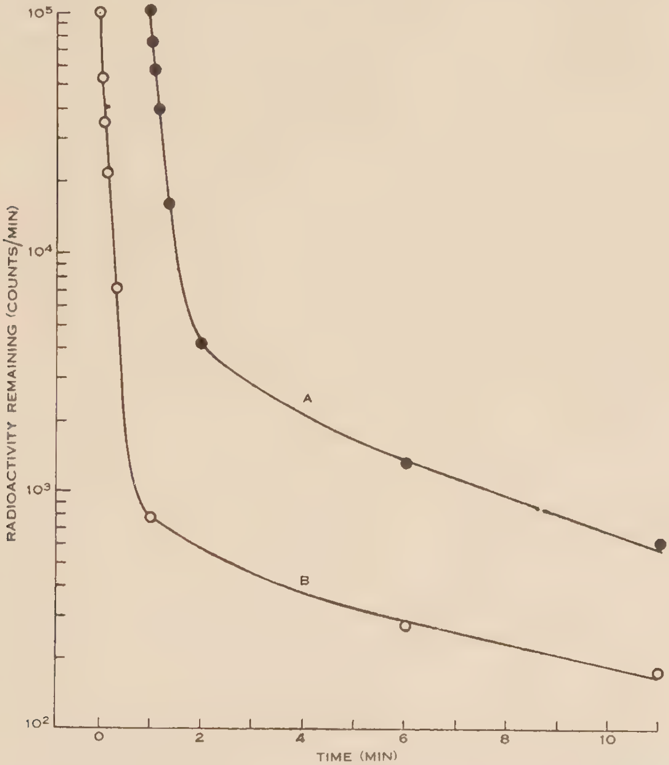


Fig. 4.—Points from Figure 3 replotted after subtraction of the extrapolates to zero time of the straight-line portions. The time scale covers only the initial 11 min. The time zeros have been separated by 1 min to prevent overlapping. *A*, cell; *B*, wall.

III. Results

(*a*) *Comparison of Exchange in Intact Cells and Walls*

The rate of loss of radioactivity from a cell which had been soaked for 24 hr in Na* (i.e. ^{22}Na) A.P.W. is shown in Figure 3, *A*. The activity remaining in the cell at various times is plotted on a logarithmic scale. The initial fast exchange was followed by a much slower loss of activity. The cell wall was then isolated and treated in the following way:

---

†NE102, Nuclear Enterprises.

(1) Soaked for a period of 2 days in inactive A.P.W.

(2) Soaked for 2 days in Na* A.P.W.

(3) Blotted, and the activity eluted into A.P.W. as before.

Figure 3, *B*, shows the time course of the release of the exchangeable sodium in the wall. The difference between *A* and *B* (Fig. 3) (25,000 counts/min), after the initial fast exchange, was in the vacuole of the intact cell, as shown by counting a sample of extracted sap. If the extrapolates of the straight-line portions are subtracted from the respective total activities, the "quickly exchangeable sodium" in intact cell and wall is as shown in Figure 4, *A* and *B*. The amounts of sodium are very similar (104,000 counts/min in the cell and 102,000 counts/min in the wall) as are the rates of the exchange ($t_{\frac{1}{2}} = 6\cdot3$ sec for the cell and $5\cdot6$ sec for the wall). The amount of quickly exchangeable sodium was in each case $0\cdot03$ μ-equiv. The volume of the cell was 75 μl.

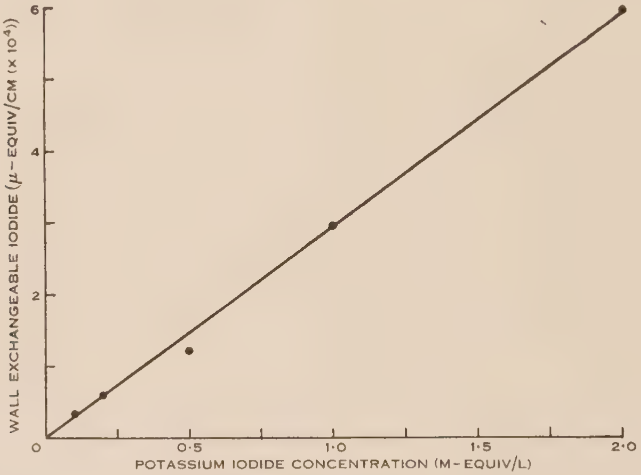


Fig. 5.—Exchangeable iodide in a *Chara australis* cell wall (in μ-equiv/cm ($\times$ 10^4)) plotted against concentration (in m-equiv/l) of potassium iodide in the external solution.

Similar results were obtained using Ca* (i.e. ^{45}Ca) A.P.W.—the intact cell and wall contained similar amounts of fairly quickly exchangeable calcium. However, the amount was greatly in excess of the exchangeable sodium, and the exchange rate much slower.

Though it is probable that there are some quickly exchangeable ions further in than the cell wall, it is clear that the majority are located in the latter and that the wall warrants a separate study.

(*b*) *Anion Exchange in the Cell Wall*

Walls were cut into 1-cm segments and one segment from each wall put into solutions of potassium iodide for 2 days. They were then transferred for 2 hr into the corresponding solution labelled with ^{131}I. The wall segments were blotted in the standard way and the activity eluted into 10 ml and counted in a liquid counter. In Figure 5 the amount of exchangeable iodide is plotted as a function of concentration.

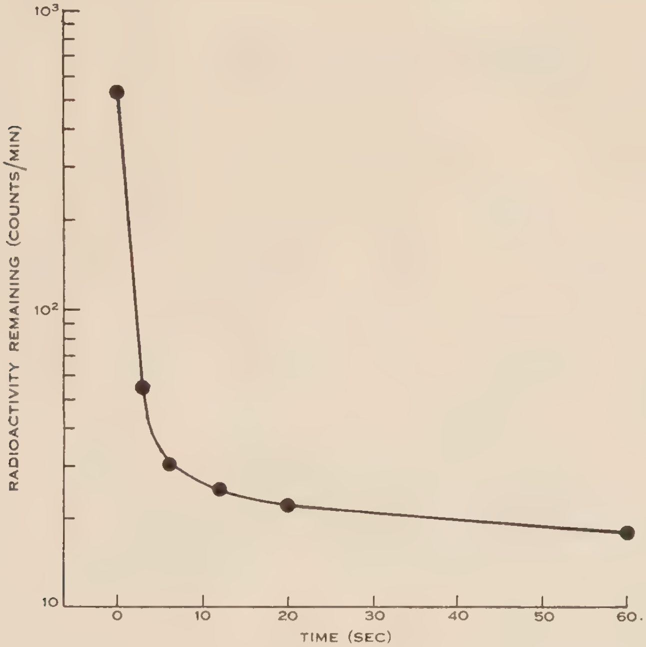


Fig. 6.—Time course of loss of radioactive iodide (in counts/min, log scale) from a *Chara australis* cell wall. The half-time for iodide exchange was 0·7 sec, and the I.F.S. 1·1 μl.

Since the amount of exchangeable iodide in equilibrium with various concentrations of anion is directly proportional to that concentration, we are dealing here with exchange with a W.F.S. (see Briggs, Hope, and Pitman 1958) or "outer space" (Epstein 1955). The mean volume of the I.F.S. in this experiment was 0·29 μl per cm length of wall, or about 0·48 μl per μl wall water.

The rate of equilibration of the wall iodide with external potassium iodide (10 m-equiv/l) is very rapid, as shown in Figure 6. A small quantity of iodide remains after 1 min but most of it leaves the wall with a half-time of about 0·7 sec. The I.F.S. was 1·1 μl (0·33 μl per μl wall water).

The fact that the I.F.S. averages about 46 per cent. of the wall water (Table 1) suggests that at least part of the wall contains a Donnan phase with a high concentration of indiffusible anions. The disposition of the I.F.S. in relation to the Donnan phase of the cell wall is discussed later.

Table 1 summarizes the data on anion exchange and I.F.S. of walls. The I.F.S. and M.F.S. have been expressed where possible in terms of μl per μl wall water by assuming a mean water content in blotted walls of 0·75 μl/mg.

Table 1

IODIDE FREE SPACE (I.F.S.) AND MANNITOL FREE SPACE (M.F.S.) AND HALF-TIMES FOR DIFFUSION EQUILIBRIUM OF IODIDE AND MANNITOL IN ISOLATED CELL WALLS OF CHARA AUSTRALIS

Expt. No.	I.F.S. (μl)	I.F.S. (μl per μl wall water)	M.F.S. (μl)	M.F.S. (μl per μl wall water)	$t_{\frac{1}{2}}$ (sec)	Blotted Weight (mg)	Length (cm)
1	2·2	0·45			1·2	6·5	6·4
			5·5	1·12	3·5		
2	1·35	0·44	—	—	1·0	4·1	5·3
3	1·1	0·49	—	—	0·7	3·0	5·0
4	1·45	(0·48)	—	—	—	—	5·0
5	—	—	3·6	1·04	—	4·6	5·1
6	—	—	3·2	0·85	4	5·0	6·0
7	—	—	2·6	(0·7)	3	—	6·5
8	—	—	3·0		2·5	—	—
Means		0·46		0·93			

(c) *The Mannitol Free Space of Cell Walls*

The release of labelled mannitol from walls into distilled water took place as shown in Figure 7 (a typical experiment). As with iodide, some label remained after 5 min but most of the mannitol diffused from the wall with a half-time of 3 sec.

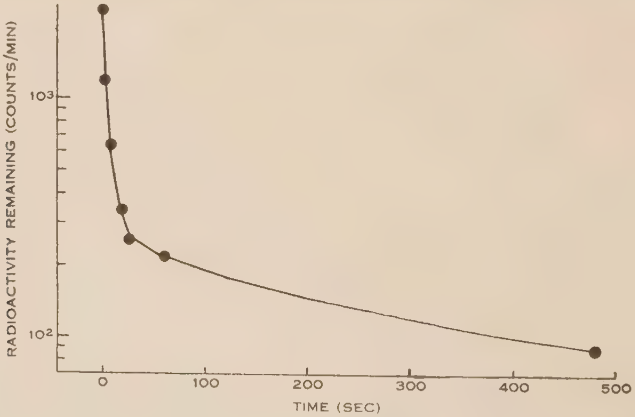


Fig. 7.—Time course of loss of radioactive mannitol (counts remaining/min, log scale), from a *Chara australis* cell wall. The half-time for mannitol diffusion was 3 sec, and the M.F.S. 5·5 μl.

The M.F.S., which is taken to correspond to the whole volume of the wall water penetrated by a molecule of this size, including the D.F.S., was 5·5 μl in this experiment. Table 1 also contains a summary of the M.F.S. of some walls, together with the half-times of equilibration.

(*d*) *Cation Exchange in the Cell Wall*

In all the following experiments cell walls were pretreated for several days in frequently renewed NaCl or $CaCl_2$ solutions to try to saturate the native anionic groups with either sodium or calcium. Then followed an equal period in labelled solution and elution into aliquots of inactive solution as described above. In the following section, the notation "Na*/1 Na" etc. means an experiment in which Na* was exchanged for Na^+ of concentration 1 m-equiv/l in the external solution.

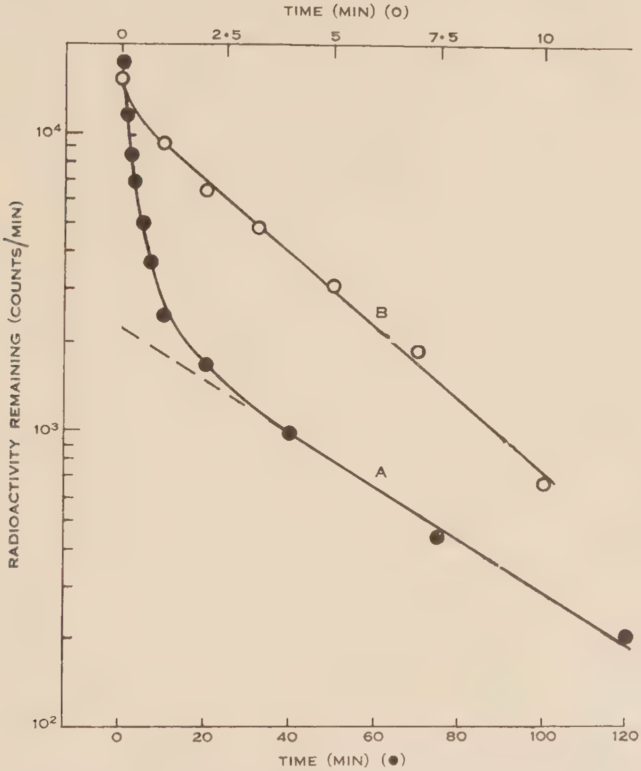


Fig. 8.—Time course of sodium exchange in a *Chara australis* wall equilibrated with sodium (counts remaining/min, log scale). *A*, total activity; *B*, fast exchange—points got by subtracting the extrapolate of the straight-line portion of *A* from the total activity at the respective times. The external concentration was 1 m-equiv/l.

(i) *Sodium Exchange.*—Figure 8 shows the results of an Na*/1 Na experiment. The total exchangeable cation was 0·225 μ-equiv. in a wall segment 2 cm long, or approximately 0·19 μ-equiv. per μl of wall water. The curve of log (radioactivity remaining) *v.* time has been divided into two parts by extrapolation of the tail of the curve (Fig. 8, *A*) to the *Y*-axis and subtraction of this extrapolate from the total. A very small fraction of the activity (10^{-3} μ-equiv.) remained in the wall after 120 min. The removal of this is not shown on the figure.

An appreciable fraction of the total activity, i.e. 0·028 μ-equiv. or 12 per cent. exchanged from the wall with a half-time of 34 min. The activity, after subtraction of the "slow" component, is plotted, on a log basis, against time (Fig. 8, *B*). The resulting graph is nearly linear with time, indicating a single exchange rate. An amount of 0·195 μ-equiv., or 87 per cent. of the total, exchanged with a half-time of 144 sec. While we do not hold the view that two (or more) discrete "compartments" are necessarily involved in sodium exchange in the cell wall, the above procedure does enable characteristic half-times to be ascribed particularly to the fastest exchange, which depend only slightly on the way in which the total curve is divided up. The conclusion from this experiment is that while some (usually the greater part) of the sodium is exchangeable with a characteristic half-time of the order of 100 sec for 1 m-equiv/l Na$^+$, the remainder is much slower to exchange, surprisingly so in view of the fact that the walls are only 16 μ thick on the average.

TABLE 2

MEAN AMOUNTS OF EXCHANGEABLE SODIUM IN ISOLATED CHARA AUSTRALIS CELL WALLS, AND MEAN HALF-TIMES OF ISOTOPIC EXCHANGE

Expt.	Total Exchangeable Sodium (μ-equiv/cm)	Fast Fraction		Other Fractions		No. of Observations
		Per Cent. of Total Exchanged	$t_{\frac{1}{2}}$ (sec)	Per Cent. of Total Exchanged	$t_{\frac{1}{2}}$ (min)	
Na*/1 Na	0·12±0·01	75±10	116±13	24±9 1	25±9 (500)	4
Na*/2 Na	0·12	70	(170)	26 4	40 (270)	1
Na*/5 Na	0·13±0·01	96±3	20±4	3±3 1	(3) (130)	3

Table 2 summarizes the half-times for Na*/Na exchange, at various concentrations. In some experiments, three segments, 2 cm long, of the same wall were put into different concentrations of inactive solution; in others, separate walls were used. The half-times are inversely proportional to the concentrations within the accuracy of the experiments. The values in parenthesis are very approximate, due to the small amounts of cation being measured, or doubtful because of some other experimental error.

(ii) *Calcium Exchange*.—Table 3 contains the results of Ca*/Ca experiments and of one Ca*/Na experiment. The isotopic exchange of calcium is somewhat slower than that of sodium, for the same concentration of the inactive (eluting) medium. The results of a typical experiment are plotted in Figure 9. The characteristic half-times of the fast fractions, which are almost unambiguous with regard to the splitting up of the total curve, are inversely proportional to the external calcium concentration.

A further feature of both sets of results is that the amount of cation in the fastest exchanging fraction tends to increase as the concentration of eluting solution is increased.

IV. DISCUSSION

The evidence given above, on the nature of the "free space" of *Chara* cell walls, can be summarized and discussed as follows:

Mannitol probably diffuses into all the wall water since the mean M.F.S. is 93 per cent. of the calculated wall water (Table 1). The I.F.S., 46 per cent. of the wall water, is less than the M.F.S. This immediately suggests some exclusion of mobile anions from part of the wall by fixed negative charges, i.e. by a Donnan system. However, the exclusion is not as great as would be expected if the complete wall were a homogeneous Donnan phase; for, from the total exchangeable cation, the average concentration of the fixed negative charge is about 0·3 μ-equiv. per μl of wall water and this would result in a mobile anion concentration of about 4×10^{-6} μ-equiv. per μl, whereas the measured mobile anion concentration is about 10^{-3} μ-equiv. per μl for an outside concentration of 1 m-equiv/l. This must mean that the wall is not a homogeneous Donnan phase but consists of a W.F.S. and a D.F.S., i.e. it must contain regions (wide pores for example, see below) which are unoccupied or only partly occupied by the electric double layer composed of the indiffusible negative ions and counterions. The suggestion that the I.F.S. exerts little Donnan effect is confirmed by the results given in Figure 5 where the exchangeable wall iodide increased linearly with external iodide concentration. This is characteristic of a W.F.S. (cf. Briggs, Hope, and Pitman 1958).

TABLE 3

MEAN AMOUNTS OF EXCHANGEABLE CALCIUM IN ISOLATED CHARA AUSTRALIS CELL WALLS AND MEAN HALF-TIMES FOR ISOTOPIC EXCHANGE

Expt.	Total Exchangeable Calcium (μ-equiv/cm)	Fast Fraction		Other Fractions		No. of Observations
		Per Cent. of Total Exchanged	$t_{\frac{1}{2}}$ (sec)	Per Cent. of Total Exchanged	$t_{\frac{1}{2}}$ (min)	
Ca*/0·5 Ca	0·14	66	1110	34	345	2
Ca*/1 Ca	0·20±0·01	52±8	321±16	39±6	54±13	4
				10±2	(800)	
Ca*/2 Ca	0·17	73	180	24	25	1
				3	(700)	
Ca*/5 Ca	0·20±0·01	87±3	84±16	8±3	22±4	4
				5±1	(550)	
Ca*/10 Ca	0·20	93	22	3	9	1
				4	(270)	
Ca*/1 Na	(0·2)	26	18000	74	15 days	1

The wall is represented schematically in Figure 10. It is possible that the I.F.S. is on the surface of the wall, i.e. consists of a film of water which is not removed by our blotting technique. This is regarded as unlikely for the film would have to be some 5 μ thick and the walls were blotted so firmly that such a film thickness could not have remained; further, the results were too consistent to be due to such an effect. From the calculated widths of the electric double layer (treated as a Gouy-Chapman layer) for the concentrations in these experiments it can be shown that the width of the macropores (W.F.S.) must be considerably greater than 100 Å to allow the presence of mobile anions. However, the wall need not contain such large-diameter pores if, in an alternative picture, one were to envisage a system of pores, all of much the same diameter, but having various amounts of fixed negative charge per unit area of pore "wall". We will usually assume, for the purposes of this discussion, a macro- and micropore system as illustrated in Figure 10 and as suggested by Northcote (1958).

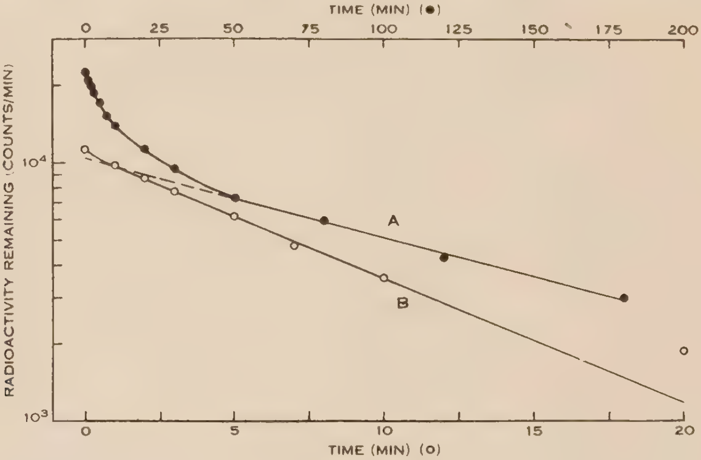


Fig. 9.—Time course of calcium exchange from a *Chara australis* wall equilibrated with calcium, plotted on a log scale. *A*, total activity; *B*, fast fraction, calculated as for Figure 8. The external concentration was 1 m-equiv/l.

Since the wall calcium can exchange completely for sodium, and from other evidence that the ion distribution obeys the Donnan distribution equations (Dainty, Hope, and Denby, unpublished data 1959), it is supposed that the forces between the cations and the exchange sites are purely electrostatic and that there is no chemical selectivity other than that due to electric charge. No chelation or other chemical bonds are envisaged.

From the mean fresh weight/cm (0·8 mg) and the data in Tables 1, 2, and 3 it can be calculated that the average amount of exchangeable sodium was 0·19 μ-equiv. per μl cell wall water, in walls in which an attempt was made to replace all the calcium by sodium. In walls in which all the counterions were made Ca^{2+}, the average amount of exchangeable calcium was 0·32 μ-equiv/μl. This difference is probably due to incomplete replacement of calcium by sodium or to less complete ionization of the indiffusible anions when Na^+ is the counterion (Briggs, Hope, and Pitman 1958; Dainty, Hope, and Denby, unpublished data 1959). If the larger value is taken as the better estimate, the average concentration of indiffusible anions in the wall is 0·32 μ-equiv. per μl of wall water. However, 46 per cent. of the total wall water is W.F.S. and therefore the true concentration of the fixed negative charge in the D.F.S. is $0 \cdot 32/0 \cdot 54 = 0 \cdot 6$ μ-equiv/μl. This value is very similar to that obtained by Briggs, Hope, and Pitman (1958) for beet storage tissue. These authors were unable to specify the location of the D.F.S. in the tissue, though they suggested the cytoplasm. In the present experiments it is quite certain that the great majority of the quickly exchangeable cations (and therefore the D.F.S.) is in the cell wall* (Figs. 3 and 4).

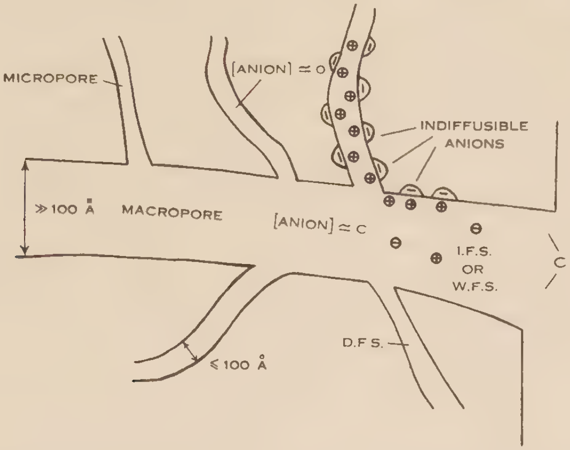


Fig. 10.—Schematic diagram of the macropore–micropore system thought to explain qualitatively the experimental results. *C*, external concentration.

It is clear from Figures 3–9 that the time course of cation exchange is complex. This complexity and the existence of an appreciable W.F.S. can be explained qualitatively by considering the wall as a system of macropores ($\geqslant$100 Å in diameter) with a system of micropores ($\leqslant$100 Å in diameter) leading into them, or by considering it as a micropore system with pore walls of widely differing charge densities or as a combination of both. Figure 10 illustrates the suggested macro- and micropore system. We can roughly identify the macropores (or, with alternative picture, the uncharged pores) with the W.F.S. and the micropores (or, alternatively, the highly charged pores) with the D.F.S. One would expect parts of the micropore (highly charged pore) system to be more difficult of access than other parts and, because of the greater hydrated size of the Ca^{2+} ion and its greater charge, calcium would have greater difficulty than sodium in getting into these parts: thus a greater fraction of the exchangeable calcium would be rate-controlled by diffusion and exchange processes in the cell wall than would be the case for sodium. This is borne out by the experimental results, which show a more complex exchange curve for calcium than for sodium. The "fast" fraction of the exchange curves is explained later as due to those fairly accessible counterions which can diffuse, in exchange for similar ions, to the surface of the cell wall faster than they can be removed to the stirred part of the external solution, by diffusion in a "stationary" film of solution. The "slowly" exchangeable ions must therefore have considerable difficulty in diffusing through the micropore (or highly charged pore) system. This can partly be explained by steric hindrance to diffusion in very narrow pores, but there also seems to be an electrostatic effect. The cations in the electric double layer due to the negatively charged walls have a smaller potential energy than those outside the double layers (this effect will be twice as large for calcium as for sodium). The activation energy for diffusion of a cation from inside to outside a double layer will therefore be greater than in free solution, i.e. the diffusion coefficient will be lower. This extra activation energy depends on the electric potential in the double layer. Our results show some experimental support for this idea for, as the external concentration is increased, the mean electric potential in the double layer is decreased and more counterions should be enabled to diffuse readily. In Tables 2 and 3 it can be seen that the percentage of total exchangeable cation in the fast fraction increases with increase in external concentration. For sodium of 1 m-equiv/l, 75 per cent. exchanges quickly, and for sodium of 5 m-equiv/l, 96 per cent. Overbeek (1956) mentions a retardation of counterions in a Donnan phase.

---

*Recent experiments by Pitman (private communication) have shown that in beet tissue, too, the D.F.S. is mostly in the cell wall.

A further possibility, which is also difficult to discuss quantitatively, is that some micropores are narrow enough to cause the filing effect discussed by Harris (1956), the counterions having to wait for a series of favourable collisions before moving along the pore. It is apparent that, under these conditions, a counterion will take longer to diffuse a given distance than when in the bulk phase.

Considering now the fast fraction, i.e. the proportion (50–96 per cent. depending on concentration) exchanging with a single rate constant, we can reasonably make the assumption that the exchange is diffusion-limited (Kitchener 1957). Now ions have to diffuse through the cell wall and then through a stationary film* of external solution before reaching the stirred solution in which we can assume that the concentration of radioactive isotope is zero. The complete solution of this problem is quite complicated (Grossman and Adamson 1952), but results can be adequately discussed by considering the two limiting cases in which the loss of the radioactive isotope is rate-controlled by (1) diffusion in a stationary film of external solution, or (2) diffusion in the cell wall itself.

If film-control is operative, then the concentration C of radioactive isotope in the wall is given by:

$$C = C_0 \exp\left(-D^s t/d\delta k\right), \qquad\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where C_0 is the concentration in the wall at time $t = 0$, D^s is the self-diffusion coefficient of the ion concerned in the *external* solution, d is the half-thickness of the flattened wall (it is assumed that the wall is washed on both sides), δ is the thickness of the stationary film, and k is the ratio of the concentration of the ion in the wall to the concentration of the ion in the external solution. Clearly log C is a linear function of time (thus justifying, in this particular case, the log-linear plotting of the results) and the half-time is given by:

---

*Such a film is better thought of as a boundary layer of laminar flow, i.e. a region where flow in a direction normal to the cell wall surface is either very small or absent. Across such a region mass flow must take place by diffusion (see Bircumshaw and Riddiford 1952).

$$t_{\frac{1}{2}} = 0{\cdot}69\,\frac{d\delta k}{D^s}. \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

For the cations Na^+ and Ca^{2+}, k is inversely proportional to the external concentration, as the cell wall is a Donnan system with a rather high fixed negative charge. Thus with film-control, the half-time should be inversely proportional to the concentration.

Tables 2 and 3 show that this is so in the present experiments for the fast fractions, within the limits of experimental accuracy. Thus most of the cation exchange between the cell wall and the external solution is, under our experimental conditions, rate-controlled by diffusion in a stationary film of external solution. A further check on this conclusion can be obtained by calculating the value of δ, the stationary film thickness, from the experimental data. Taking an average value for d, the cell wall half-thickness, of 16 μ, the average sodium concentration in the cell wall as 130 m-equiv/l of cell wall and the calcium concentration as 217 m-equiv/l of cell wall, the values of δ calculated from equation (2) are 90 μ from the sodium results and 106 μ from the calcium results. The order of magnitude of these results is reasonable for the stirring conditions used (Bircumshaw and Riddiford 1952; Tetenbaum and Gregor 1954) and the agreement between the two values of the stationary film thickness is strong confirmation of the film-control hypothesis. A further deduction can be made; if the sodium exchange is film-controlled, the half-time for exchange of sodium between an untreated cell wall and its normal environment (A.P.W.) can be calculated from the results on "pure sodium" walls. This half-time works out to be 10 sec, in rough but adequate agreement with the experimental value of 5·6 sec (wall with rest of cell) or 6·3 sec (wall isolated)—see Section III(*a*). The exchange of calcium between the untreated cell wall and A.P.W. proceeds, of course, at the same rate as between calcium-treated cell wall and pure 0·5 m-equiv/l $CaCl_2$ solution, because Ca^{2+} is the chief counterion in the D.F.S. of the untreated wall. Thus in the normal cell wall in A.P.W., calcium exchanges about 25 times more slowly than sodium.

The average half-time for exchange of iodide between A.P.W. and the cell wall was 1·0 sec and that for loss of mannitol was 3·3 sec. While film-control is probably operative, equation (1) does not apply to the diffusion of iodide and mannitol, since the capacity of the laminar layers is comparable with that of the I.F.S. or M.F.S. (The capacity of the laminar layers for cations has been neglected in comparison with that of the D.F.S.) Thus it is not possible to deduce any quantitative information about iodide and mannitol exchange. However, from Figures 6 and 7 it can be seen that not all the iodide and mannitol leaves the wall with a single rate constant. The small amounts slow to exchange may be hindered in the micropores (mannitol) while the same may apply to iodide provided it penetrates some of the larger micropores.

To summarize, the results of this study thus indicate that the apparent free space of *Chara australis* is located in the cell wall and comprises both a W.F.S. and a D.F.S.; the exchange of ions, particularly cations, between free space and external solutions of "physiological" concentrations is mainly rate-limited by diffusion in a stationary film of external solution of effective thickness about 100 Å. Presumably the free space in *Nitellopsis obtusa*, studied by MacRobbie and Dainty (1958), is also entirely confined to the cell wall; they identified in this species an apparent free space, a protoplasmic non-free-space, and the vacuole as three separate compartments, but were unable to say whether or not part of the protoplasm might be in the apparent free space compartment. A recent study by Diamond and Solomon (1959) on "intracellular potassium compartments in *Nitella axillaris*" is in general agreement with the results of MacRobbie and Dainty (1958) and, in addition, proves that the potassium free space is entirely in the cell wall, which constitutes a Donnan system. They have further proved that the cell wall, the protoplasm, and the vacuole are, kinetically, "in series".

In our experiments on *Chara australis*, it has so far not been possible to distinguish unambiguously a protoplasmic non-free space in the time course of exchange of sodium or calcium in an intact cell. This is partly because of the complex nature of the cation exchange in the cell wall, but studies with ^{42}K—when it becomes available in Australia—should resolve this difficulty. In the past, the cytoplasm has been included in the free space, notably by Briggs (1957), Briggs and Robertson (1957), and Briggs, Hope, and Pitman (1958); others have considered that a plasmalemma exists at the surface of the cytoplasm, and this controls ion transport into the cytoplasm (Levitt 1957; Walker 1957; MacRobbie and Dainty 1958). It would appear from the present study that the free space, including the D.F.S. of *Chara australis* is located entirely in the cell wall, which must contain substances, presumably "pectins", which can ionize to give rise to fixed (Donnan) anions (Dainty, Hope, and Denby, unpublished data 1959). The protoplasm also, of course, contains indiffusible anions, but it does not seem to contribute to the D.F.S.; the reason for this may lie in the control of ion transport into the protoplasm by a plasmalemma.

V. ACKNOWLEDGMENTS

Thanks are due to Dr. R. N. Robertson, Mr. E. W. Hicks, and Dr. J. H. B. Christian, Division of Food Preservation and Transport, C.S.I.R.O., for their helpful criticisms of the manuscript and to Dr. J. R. Vickery, Chief, Division of Food Preservation and Transport, C.S.I.R.O., and Professor R. L. Crocker, Professor of Botany, University of Sydney, in whose Laboratories the work was carried out.

One of us (J.D.) gratefully acknowledges receipt of a Royal Society–Nuffield Commonwealth Bursary.

VI. REFERENCES

BIRCUMSHAW, L. L., and RIDDIFORD, A. C. (1952).—Transport control in heterogeneous reactions. *Quart. Rev.* **6**: 157.

BRIGGS, G. E. (1957).—Some aspects of free space in plant tissues. *New Phytol.* **56**: 305.

BRIGGS, G. E., HOPE, A. B., and PITMAN, M. G. (1958).—Exchangeable ions in beet disks at low temperature. *J. Exp. Bot.* **25**(9): 128.

Briggs, G. E., and Robertson, R. N. (1957).—Apparent free space. *Annu. Rev. Pl. Physiol.* **8**: 11.

Diamond, J. M., and Solomon, A. K. (1959).—Intracellular potassium compartments in *Nitella axillaris*. *J. Gen. Physiol.* **42**: 1105.

Epstein, E. (1955).—Passive permeation and active transport of ions in plant roots. *Plant Physiol.* **30**: 529.

Grossman, J. J., and Adamson, A. W. (1952).—The diffusion process for organolite exchanges. *J. Phys. Chem.* **56**: 97.

Harris, E. J. (1956).—"Transport and Accumulation in Biological Systems." p. 37. (Butterworths Scientific Publications: London.)

Hope, A. B. (1953).—Salt uptake by root tissue cytoplasm: the relation between uptake and external concentration. *Aust. J. Biol. Sci.* **6**: 396.

Hope, A. B., and Stevens, P. G. (1952).—Electric potential differences in bean roots and their relation to salt uptake. *Aust. J. Sci. Res.* B **5**: 335.

Kitchener, J. A. (1957).—"Ion-Exchange Resins." pp. 49–60. (Methuen: London.)

Levitt, J. (1957).—The significance of "apparent free space" (A.F.S.) in ion absorption. *Physiol. Plant.* **10**: 882.

MacRobbie, Enid A. C., and Dainty, J. (1958).—Ion transport in *Nitellopsis obtusa*. *J. Gen. Physiol.* **42**: 335.

Middleton, L. J., and Russell, R. S. (1958).—The interaction of cations in absorption by plant tissues. *J. Exp. Bot.* **25**: 115.

Northcote, D. H. (1958).—The cell walls of higher plants: their composition, structure, and growth. *Biol. Rev.* **33**: 53.

Overbeek, J. T. (1956).—The Donnan equilibrium. *Progr. Biophys. & Biophys. Chem.* **6**: 57.

Sutcliffe, J. F. (1957).—The selective uptake of alkali cations by red beet tissue. *J. Exp. Bot.* **8**: 36.

Tetenbaum, M., and Gregor, H. P. (1954).—Self-diffusion of cations, non-exchange anions, and solvent in a cation-exchange resin system. *J. Phys. Chem.* **58**: 1156.

Walker, N. A. (1957).—Ion permeability and the plasmalemma of the plant cell. *Nature* **180**: 94.

STUDIES OF ACTION POTENTIALS IN THE VACUOLE AND CYTOPLASM OF *NITELLA*

By G. P. Findlay*

[*Manuscript received May* 14, 1959]

Summary

Intracellular microelectrodes have been used to record the action potential in the vacuole and cytoplasm of single cells of the alga *Nitella*. The *Nitella* cells were cultured in White's nutrient solution ($9 \cdot 2 \times 10^{-3}$ g total salts/l) and almost all measurements of the action potential were made on cells in this solution. The resting potentials of the vacuole and cytoplasm with respect to the bathing solution are approximately equal, and have a value of -150 mV.

The action potential in the vacuole, of 93 mV magnitude, was initiated by a short ($0 \cdot 1$ sec) depolarizing pulse of electric current. Two components—a fast component (the spike) of approximately 2 sec duration and a slow component (superposed on the fast component) lasting 5–10 sec, are identified in the action potential.

The action potential in the cytoplasm is almost identical with the action potential in the vacuole. Thus it appears that the action potential occurs almost entirely across the outer cytoplasmic boundary (the plasmalemma).

The effects of long depolarizing and hyperpolarizing current pulses on the vacuole potential have been examined. Measurements of the resistance of the resting cell have been made by passing electric current pulses between vacuole and bathing solution, and recording the change in vacuole potential. The resistance measured with a depolarizing current is less than the resistance measured with a hyperpolarizing current.

I. Introduction

Although extensive investigations have been carried out on excitatory processes in living cells for more than half a century, it has only been in the last two decades that considerable advances in the explanation of the behaviour of such cells have been made. Not surprisingly, much more has been done in the study of animal nerve cells than has been done in plant cells. Such developments as electronic measuring equipment and intracellular microelectrodes have enabled a very full investigation of many excitatory phenomena, in nerve cells, to be made. A most notable example is the work of Hodgkin, Huxley, and Katz (1952) on the squid axon. However, corresponding advances have not been made for plant cells such as the alga *Nitella*, which exhibit properties similar to those of nerve cells.

The large internode of *Nitella* has long been a subject for studies in excitatory phenomena, but, up to the present, only the action potential in the vacuole has been recorded. The earliest measurements of the action potential (Osterhout 1931, 1934,

---

* Physics Department, University of Tasmania.

1936, 1944; Hill and Osterhout 1934, 1938) were made by a method in which electrical contact with the vacuole was obtained by killing a region of the cell with chloroform. Cole and Curtis (1938) have also used this method. Although convenient, there is an obvious objection to the method in that the *Nitella* cells only survived for a few hours after such treatment, and were probably in an abnormal condition during the experimental period. Umrath (1930, 1932*a*, 1932*b*, 1934, 1953, 1954), on the other hand, inserted fine glass capillaries (filled with an electrical conducting medium) into *Nitella* cells and was able to measure the vacuole potential without unduly disturbing the cell. Recently Oda (1956) has made measurements of the action potential in *Chara braunii*, using intracellular microelectrodes.

This paper describes the results of measurements of the action potential in *Nitella*, both in the vacuole and in the cytoplasm. These measurements have been made with intracellular microelectrodes similar to those used by Walker (1955) and with improved electrometers and pen-recording equipment.

It is found that the action potential in the vacuole is almost identical with the action potential in the cytoplasm. This potential change is thus located at the outer cytoplasmic boundary, the plasmalemma. As shown by Walker (1955), and confirmed here, the resting potential of the cell is also located at the plasmalemma.

It is known that there is a close relationship between the cytoplasmic streaming and the action potential (Hill 1941). Further observations on this relationship are described in this paper.

Considerable care has been taken to standardize the experiments as far as possible, and so obtain reproducible results from a series of cells. Previous workers did not seem to be greatly concerned with maintaining standard culture methods. For example, Oda (1956) gives results for action potentials in *Chara braunii* bathed in tap water, but does not give the ionic constitution of this bathing solution nor of the original culture solution. A knowledge of these ionic constituents is essential if any theory of the action potential is to be formulated. In this paper, a new and satisfactory method of culturing *Nitella* under controlled conditions is described.

II. Materials, Apparatus, and Methods

In some preliminary experiments, *Nitella* cells of an unknown species were used which had been grown in stagnant cultures. These cultures consisted of a fine river gravel base and tap water (which was changed occasionally). However, it was found that reproducibility of results from cell to cell, and from culture to culture, could not be obtained. Furthermore, the ionic content of each culture solution was not known and was variable. To counteract these difficulties a new and more satisfactory culture method has been evolved. Old-stock cells (grown in a glass-house pond) were placed in a "Perspex" dish, 10 cm deep, and covered to a depth of 1 cm with white quartz gravel. This gravel had been previously treated with boiling distilled water for several days until the diffusion of salts from the gravel was negligible as evidenced by conductivity measurements. A culture solution, similar to that described by White (1943), flowed through the culture bath and maintained a constant environment for the *Nitella* cells. The ionic content of this culture solution is shown in Table 1. A new growth of cells appeared in 10–14 days. The cultures were kept indoors at 20°C and given 15 hr light per day. These conditions approximated to the most extreme summer conditions that the plant would meet in its natural environment and were chosen to give maximum growth rate. This produced cells which were sufficiently transparent to enable inserted microelectrodes to be seen clearly.

The experimental cell (usually 1·5 cm long) was held on a "Perspex" mount with small pieces of clean petroleum jelly. In almost all experiments described in this paper the cell was bathed with White's solution of the same concentration as the culture medium, flowing continuously at a rate of 2 c.c./min to ensure a constant environment for the cell. It was decided that action potentials be measured in cells of *Nitella* in their culture medium because the main purpose of the investigations described in this paper was to ascertain the nature of the action potential of *Nitella* in its normal state before any measurements are made with changed external conditions.

Table 1

IONIC CONSTITUENTS OF MODIFIED WHITE'S CULTURE SOLUTION

Ion	Concentration (μ-equiv/l)	Ion	Concentration (μ-equiv/l)
Mg^{2+}	600	SO_4^{2-}	890
Ca^{2+}	240	NO_3^-	200
Na^+	150	Cl^-	90
K^+	170	PO_4^{3-}	40
Mn^{2+}	3·0	BO_3^{3-}	7·8
Zn^{2+}	0·9	I^-	0·45

Measurements of the electric potential of the vacuole and cytoplasm of the *Nitella* cell were made with "Pyrex" glass microelectrodes or probes (filled with 0·3N KCl as a conducting medium) similar to those described by Walker (1955). The microelectrodes (of tip diameter between 5 and 8 μ and resistance between 20 and 5 MΩ) were inserted into the *Nitella* cell using fine screw manipulators. Both single- and double-barrelled probes have been used. The double-barrelled probes were made by fusing together, side by side, two pieces of glass tubing (6 mm external dia.), and then drawing them out, first in a gas flame, and then on a de Fonbrune microforge, as is done with single probes. Symmetrical calomel half-cells connected the solution in the probe and a reference point in the bathing solution to the input of one of the electrometers.

The action potential in *Nitella* is initiated when the vacuole potential is depressed below a critical level. This depression can be caused by:

(1) The passage of an electric current from the vacuole to the bathing solution (Osterhout 1931).

(2) An increase in ionic concentration of the external solution (Osterhout 1931).

Action potentials can also be caused by:

(3) A sudden decrease in temperature (Osterhout and Hill 1935).

(4) Mechanical shock (Osterhout 1931).

Of these methods, (1) has been used for the experiments described in this paper. This method of stimulation can be controlled much more easily than other methods, and allows very rapid changes to be made in the plant potential. Generally, in measurements of the action potential, stimulating current pulses of 0·1 sec duration and magnitudes of $10^{-7} - 5 \times 10^{-6}$ A cm^{-2} of cell membrane have been used. This pulse time has been chosen because the stimulating current is removed very soon after the action potential commences and consequently does not confuse the records of the action potential obtained from the plant cell itself. With current pulses shorter than 0·1 sec, there is considerable variability in the strength of pulse needed to initiate the action potential, and consequently these short pulse times have not been used in the present investigation. In Sections III(*c*) and III(*d*) observations have also been made of the action potential with stimulating current pulses lasting several seconds. The cell was stimulated once every 10–15 min. This time was chosen to allow the resting potential to return fully to its normal value, and the cytoplasmic streaming rate to reach its maximum. If the interval between stimulation was 5 min or less, the action potential changed in form after several stimulations.

The stimulating current was applied to the cell through a probe inserted into the vacuole. A high impedance (50 MΩ) potential source was connected to this probe, and to a platinum plate in the bathing solution. The high impedance ensured a constant current flow through the stimulating probe irrespective of changes in cell potential and resistance. The potential source supplied rectangular current pulses of varying current strength and duration. The potential drop across a 10 kΩ resistance in series with the stimulating probe was fed into an electrometer, which enabled the stimulating current to be measured.

In measurements of the vacuolar action potential two probes were inserted into the vacuole of the cell—the stimulating probe and a recording probe.

The action potential in the cytoplasm was measured in two ways. First, two probes (a single and a double probe) were inserted into the cell. The double probe was inserted 150–200 μ into the vacuole. One barrel of this probe recorded the vacuole potential and the other barrel acted as a stimulating probe. The single probe was inserted 20–40 μ into the vacuole. Shortly after the insertion, the cytoplasm flowed up and over the probe and at the same time, the seal began to form on it (cf. Walker 1955). By moving this probe a little further into the cell every 10 min or so, the tip could be kept clear of the seal and yet remain in the cytoplasm. This movement did not stimulate the cell. The probe could be kept in the cytoplasm for 1–2 hr in this way. In the second method, the single probe was inserted and then pushed to the far side of the *Nitella* cell, until its tip (as seen under a magnification of × 600) was in the cytoplasmic stream. As long as this probe does not touch the cell wall it will not seal and it can be moved at will from cytoplasm to vacuole and vice versa.

The electrometers, of a new design, were constructed at this University. They had an input impedance of 10^{10} ohms and were characterized by a high degree of stability. On the range 0–10 mV the zero drift was less than 0·5 mV during a 12-hr run. This stability was obtained by the use of an extremely stable potential supply for the filaments of the two input electrometer valves. The D.C. input to the electrometer was applied to the grids of these valves (ME 1400), the screens of which were driven at 1000 c/s, so that the output of this stage was a 1000 c/s signal of amplitude dependent on the D.C. input level. The rectified output of the amplifier (max. 30 V, 10 mA) was fed into an Evershed and Vignoles high-speed pen recorder (chart speed usually 0·25 or 0·5 in/sec). A block diagram of the experimental set up is shown in Figure 1.

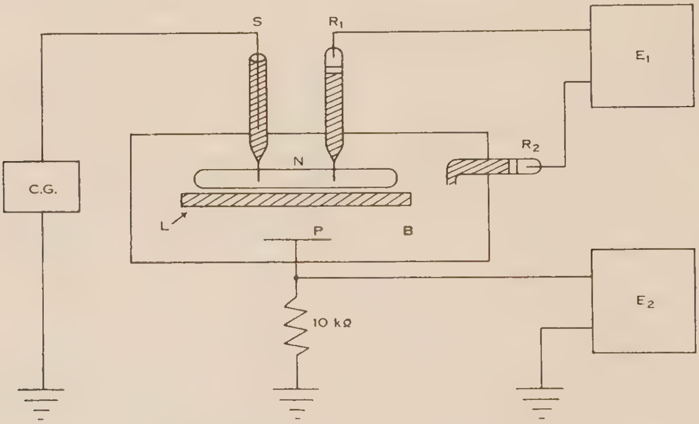


Fig. 1.—Diagram of the experimental set-up (shown in plan) for measuring the action potential in the vacuole. *N*, *Nitella* cell; *S*, stimulating probe; R_1, recording probe; R_2, reference electrode; *L*, small ledge to prevent movement of the *Nitella* cell when the two probes are inserted; *B*, bathing solution; *P*, platinum plate; *C.G.*, stimulating current generator; E_1, electrometer measuring plant potential; E_2, electrometer measuring stimulating current. The cell is observed with a microscope using a water-immersion objective.

III. RESULTS

(*a*) *The Action Potential of* Nitella *Cells in Culture Solution*

(i) *The Action Potential in the Vacuole.*—The action potential (its form being shown in Fig. 2) has been measured in 26 cells in the solution in which they were grown (White's nutrient solution, see Table 1). None of these cells was more than 6 weeks old, and their mean vacuole resting potential was 150 mV with a standard deviation of 5 mV. A depolarizing current pulse of 0·1 sec duration was applied to the cell through a stimulating probe causing the vacuole potential to become less negative. This applied current pulse is called suprathreshold or subthreshold depending on whether or not an action potential is initiated. When the threshold has been passed the action potential is initiated, and will then proceed independently of the stimulating current. The action potential is characterized by a rapid rise (maximum rate 194 ± 46 mV/sec) in the vacuole potential to a mean value of —50 ± 15 mV, and then a recovery of this potential at a slower rate, to the original resting level. The maximum change in vacuole potential is 93 ± 11 mV. The least negative value of the vacuole potential was reached at a time between 0·5 and 2·0 sec after the stimulating current was applied.

Figure 3 shows action potentials recorded in four cells. Each record is formed by the superposition of several traces obtained from the one cell. It can be seen that in any one cell there is not a great deal of scatter in the action potential traces. However, there is some variation from cell to cell. This is thought to be due mainly to differences in age of cells used.

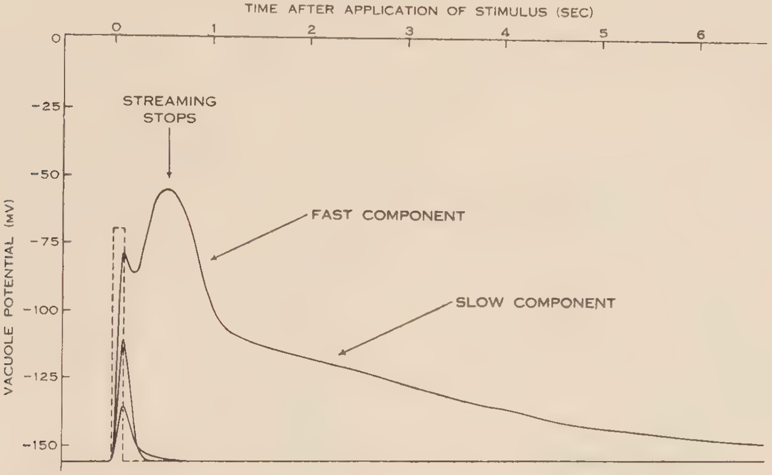


Fig. 2.—The action potential measured in the vacuole of a *Nitella* cell. The response of the vacuole potential to two subthreshold pulses and a suprathreshold pulse is shown. The broken line shows the potential applied between the vacuole and the bathing solution by the stimulating current. The two components in the action potential are indicated, as well as the time of stoppage of streaming.

Two components can be identified in the action potential—a fast component and a slow component (Fig. 2). The fast component (which will be called the "spike") is of approximately 1–3 sec in duration, and appears as the fast rise and fall of the vacuole potential which occurs once the action potential has been initiated. The slow component (lasting 5–10 sec) is superposed on the fast component. The decline of this component takes the vacuole potential (from an intermediate value of —100 mV to —120 mV) back to its original resting level.

Results from three cells, which were probably older than most cells used, showed action potentials in which there was a clear separation of the two components. The fast component had occurred normally, but the slow component had been delayed for a short time (Fig. 4).

A few measurements have also been made of the action potential in cells older than 2 months. In these cells it is possible to elicit action potentials of much smaller magnitude with stimuli at, or just above, threshold. Strong stimuli, however, cause the normal action potential. This is in contrast to the behaviour of young cells, where a stimulating pulse, if it causes an action potential at all, will always initiate an action potential of the normal magnitude.

(ii) *The Action Potential in the Cytoplasm.*—Action potentials have only been recorded in the cytoplasm of young cells 2–3 weeks old because older cells were not sufficiently transparent to allow observation of the probe tip. Measurements have been made with probes in the cytoplasmic stream adjacent to the point of probe insertion, and in the stream opposite to the point of probe insertion. In both cases records have been obtained from five cells. As only one electrometer channel was available when these measurements were made, the action potential was recorded alternatively, at 10–15-min intervals, first by the probe in the vacuole and then by the probe in the cytoplasm. By comparing these two measurements it was found that the action potential in the cytoplasm was almost identical with the action potential in the vacuole. There was no significant difference between the heights of the spikes, measured in the cytoplasm and in the vacuole. Although some scatter was apparent in the records of the action potential, particularly in the recovery (the slow component) where the scatter was as large as 15 mV in both the cytoplasm and vacuole measurements, there was no consistent difference between the action

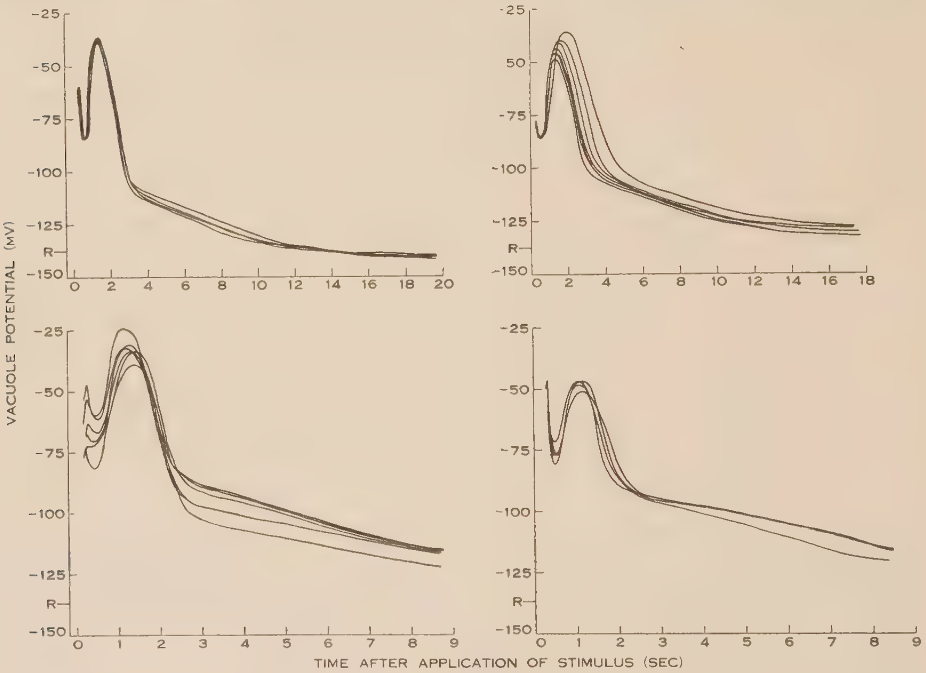


Fig. 3.—Action potentials recorded in the vacuoles of four cells. In the record for each cell, a number of action-potential traces have been superposed. All action potentials have been initiated with a 0·1 sec current pulse. The vacuole potential before the stimulus is applied is shown by the line *R* to the left of the potential axis. Note that the time scale for the two lower traces differs from the scale for the two upper traces.

potential traces in these two regions. That is, there was no consistent potential difference across the tonoplast during the action potential.

(iii) *The Action Potential in Relation to Stoppage of Cytoplasmic Streaming.*—A series of observations on *Nitella* cells in White's culture medium has shown that in these cells the cytoplasmic streaming, so characteristic of all members of the Characeae, stops abruptly when an action potential occurs. For a wide range of stimulus strength, the time when the streaming stops corresponds closely to that of the top of the spike. It appears that the stoppage of streaming is in some way dependent on the occurrence of the action potential, and not caused directly by the application of a stimulus. Even in cases where a long current pulse is applied to the cell (see Section III(*c*)(i)) and the action potential is delayed for many seconds after the pulse has commenced, the streaming has always stopped at the top of the spike.

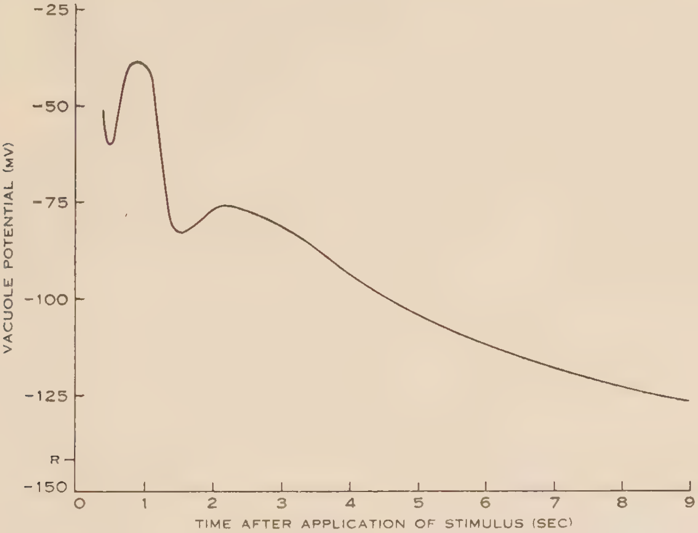


Fig.4.—Record of an action potential in which the fast component and the slow component are clearly separated.

An action potential occurring in a cell in which the cytoplasm is streaming always causes the streaming to stop. No exceptions have been noted in approximately 1500 action potentials observed. On the other hand, action potentials can be initiated in cells in which the cytoplasm is stationary. Subthreshold depolarizing current pulses and hyperpolarizing current pulses both lasting up to 30 sec do not stop streaming although they may slow it down (cf. Hill 1941). Usually the cytoplasm has regained its original streaming rate 2–3 min after an action potential has occurred.

(*b*) *The Action Potential in* Nitella *Cells in Potassium Chloride*

Before measurements were made of the action potential in *Nitella* in its White's culture medium, a preliminary investigation had been made of action potentials in cells, cultured in White's solution, and transferred to 0·0001N KCl about 24 hr before the experiment began. During the course of an experiment lasting 3–4 hr, in which the cell was stimulated every 10–15 min, the shape of the action potential in the vacuole changed, and its magnitude declined to less than 60 per cent. of the original value, even though the resting potential remained approximately constant This behaviour contrasts with the behaviour of the cell in its culture medium, where the action potentials do not change. It is to be noted that when the solution of 0·0001N KCl bathing the cell was changed back to White's solution the action potential changed to its normal form within 1 hr.

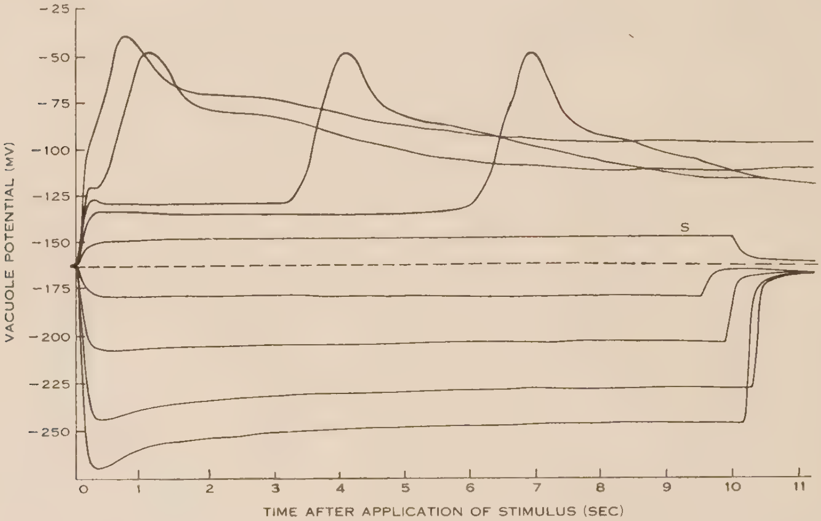


Fig. 5.—Response of the vacuole potential to the application of long depolarizing and hyperpolarizing current pulses of different intensities. The broken line shows the resting potential of the cell. The traces above this line show the response to depolarizing pulses. The curve *S* is a subthreshold response and the four upper curves suprathreshold responses. As the strength of the applied current pulses increases, the action potentials occur at decreasing time intervals from the start of the pulse. The traces below the broken line show the responses to hyperpolarizing pulses of increasing strengths—the strongest pulse producing the largest change in vacuole potential. The hyperpolarizing current pulses have terminated approximately 10 sec after application, but the depolarizing pulses (except the subthreshold pulse) still continue after 11 sec. In this cell, the vacuole potential did not overshoot its resting level when the hyperpolarizing pulse terminated.

(c) *The Effect of Long Current Pulses*

All measurements described in this section were made on cells bathed in White's culture solution.

(i) *Depolarizing Current Pulses.*—Depolarizing current pulses of several seconds duration have been passed between the vacuole and the bathing solution. These pulses, providing they were subthreshold, produced a steady change in the vacuole potential while suprathreshold pulses produced the characteristic action potential.

Measurements were made on 13 cells. Figure 5 shows the potential changes recorded in one of these cells when long current pulses ranging in strength from subthreshold to suprathreshold were applied. It was found that for suprathreshold pulses the time between commencement of the pulse and the top of the spike depended on the strength of the pulse; the stronger the stimulus the sooner the action potential occurred.

An investigation of the time, Δt, between the start of a stimulating current pulse (maintained throughout the action potential) and the top of the spike, and the current density I, yielded a relationship of a hyperbolic nature (Fig. 6). The asymptote intersecting the current density axis indicates the minimum current density which will produce an action potential, i.e. the threshold current density for long current pulses. For current densities just above this value it is possible to obtain large values of Δt, some being as great as 15 sec. It can also be seen that as pulse strength increases, Δt decreases to a minimum value. This means that there is a limiting time for the action potential to occur, no matter how great the stimulating current. For long current pulses, the threshold potential (the potential the vacuole takes when the stimulating current of threshold intensity is applied) is much closer to the resting potential than is the case for stimulating current pulses of 0·1 sec duration.

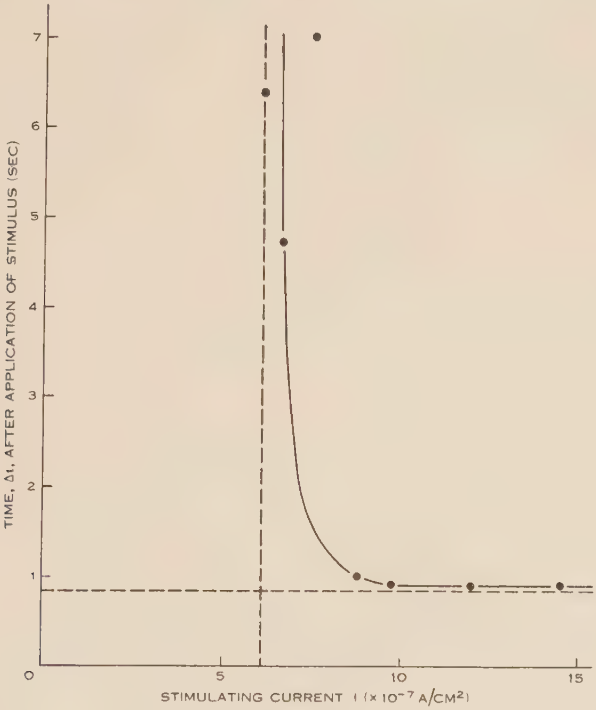


Fig. 6.—Relationship between the time, Δt, from the start of the stimulating current to the top of the spike and the current intensity, I.

In some experiments on old cells, it was found that long current pulses at and just above threshold caused the slow component of the action potential to appear alone. Slightly stronger stimuli caused the slow component to begin by itself, but very soon afterwards the fast component appeared also. With strong stimuli the action potential pattern is normal.

(ii) *Hyperpolarizing Current Pulses.*—In a series of experiments, on 13 cells, rectangular hyperpolarizing electric current pulses of several seconds duration were applied to the cell (Fig. 5). Certain features of the response of the vacuole potential to the hyperpolarizing current are characteristic. First, when the current flow commences, the vacuole potential changes (approximately exponentially) to a potential more negative than the resting potential, but then declines to a potential closer to the resting potential. Secondly, when the hypèrpolarizing pulse terminates the potential rises exponentially towards the resting level, but in some cases overshoots this level before finally recovering. If, in the case of overshooting, the potential rises above the threshold for stimulation, an action potential occurs.

(*d*) *The Cell Resistance*

Measurements have been made of the resistance between the vacuole and the bathing solution (White's) just outside the cell. Measurements were made on 6 cells. Long subthreshold depolarizing and long hyperpolarizing current pulses of low intensity were applied between the vacuole and the bathing solution and the change in vacuole potential recorded.

While the current flows, the potential recorded by a probe in the vacuole is the sum of the plant potential and the ohmic potential drop across the resistance between the vacuole and the bathing solution. A correction is made for the potential across the bathing solution in the following way. Before the recording probe is inserted into the cell, its tip is placed just outside the cell, in the bathing solution. Electric current is passed by the inserted stimulating probe, and a potential change is shown by the recording probe. The recording probe is then inserted into the cell, and further measurements made. The difference between the internal and external potential measurements is the potential change between the vacuole and a point just outside the cell caused by the current flow.

The cell resistance was defined as the ratio of the potential change to the magnitude of the applied current. For depolarizing current pulses the resistance was constant during the current flow. For changes in vacuole potential up to 40–50 mV, the resistance is approximately linear. For six cells, the mean value of the resistance was $(2\cdot7 \pm 1\cdot2) \times 10^4\,\Omega\,\mathrm{cm}^2$.

However, for hyperpolarizing constant current pulses, the vacuole potential varies during the application of the pulse (Fig. 5). Nevertheless, the cell resistance defined in terms of either the initial or the final potential change is greater than the resistance determined with a depolarizing current. The resistance defined in terms of the final potential change has a value $(4\cdot6 \pm 1\cdot2) \times 10^4\,\Omega\,\mathrm{cm}^2$. Figure 7 shows the relationship between the change in vacuole potential, and the applied current, an abrupt change of slope appearing at the resting potential.

Some preliminary investigations have also been made of the change in total cell resistance during an action potential. A suprathreshold current pulse was applied to the cell. This pulse was turned on and off four times per second. At this frequency capacitive effects of the membrane are negligible. The amplitude of the oscillation of the vacuole potential so obtained is the potential drop between the vacuole and the bathing solution, caused by the flow of the applied current of constant amplitude and consequently is proportional to the resistance between the vacuole and bathing solution. A correction can be made for the resistance of the bathing solution, and thus the cell resistance can be determined. The resistance decreases greatly during the rising phase of the action potential and recovers to approximately its normal value before the slow component of the action potential starts to decline. However, accurate measurements of the minimum resistance are difficult to obtain, because the amplitude of the potential oscillation becomes very small when the cell resistance decreases. From measurements made on six cells, it was found that the cell resistance decreased to possibly less than 1 per cent. of the resting resistance (cf. Cole and Curtis 1938), although not much weight is given to this value. The

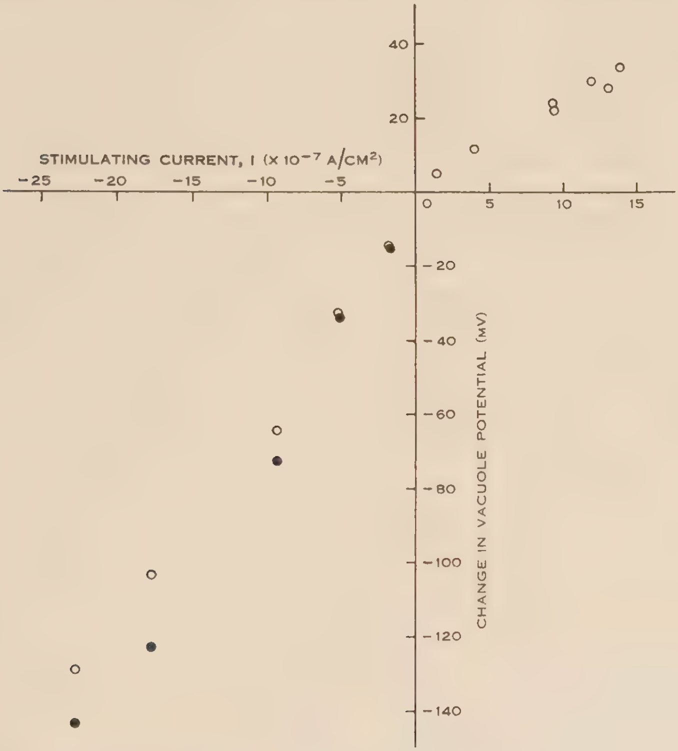


Fig. 7.—Relationship between current flowing between vacuole and bathing solution, and the change in vacuole potential. Depolarizing current is taken as positive. ○ Final change in the vacuole potential. ● Initial change in vacuole potential when a hyperpolarizing current is applied.

use of a voltage clamp method (Hodgkin, Huxley, and Katz 1952) for measuring the cell resistance during activity is at present under consideration.

IV. Discussion

In Section III it was seen that the action potential in a *Nitella* cell is located at the outer cytoplasmic boundary—the plasmalemma. Walker (1955) has shown that the resting potential also is located at the plasmalemma. Thus, when the cell is in both resting and active conditions, practically no potential difference is maintained, or appears, across the inner cytoplasmic boundary—the tonoplast. These results make untenable the theory put forward by Osterhout (1927, 1934) to account for the action potential in *Nitella*, in which it was assumed that the major part of the resting potential of the cell was located at the tonoplast, and that this potential changed during activity.

It appears that the tonoplast does not play a direct role in the steady and transient electrical behaviour of the cell. Its importance is most probably in relation to ion-carrier processes. For *Nitellopsis obtusa*, MacRobbie and Dainty (1958) have suggested that a Cl^- pump is located at the tonoplast. Although the *Nitella* used in experiments described in this paper grows in fresh water, while *Nitellopsis obtusa* grows in brackish water, an analysis of vacuolar sap, made in this Laboratory and elsewhere shows that Cl^-, and probably other ions, are not in electrochemical equilibrium between the vacuole and outside medium, and consequently need to be actively transported into the vacuole. It is likely that the tonoplast is involved in such transport.

MacRobbie and Dainty (1958) have also shown that in *Nitellopsis obtusa* the resting flux of K^+, Na^+, and Cl^- ions across the tonoplast is 20–100 times lower than the flux of these ions across the plasmalemma. A similar situation probably obtains in the *Nitella* used in experiments described in this paper. As also shown in this paper, the action potential occurs across the plasmalemma, and consequently involves considerable changes in ionic fluxes across this membrane but probably not across the tonoplast. Hence, the tonoplast appears to play little direct part in the short term electrical changes which comprise the action potential. One is thus led to the conclusion that a three-phase system, consisting of cytoplasm–plasmalemma–outside medium, controls the transient electrical behaviour of the cell. Unfortunately, very little information on the ionic composition of the cytoplasm is available.

In Sections III(*a*) and III(*b*) it was seen that in *Nitella* cells in their culture medium the action potential has two components. In young cells both components always occur, but in some older cells it is possible to obtain the slow component by itself. It is possible that future investigations will be able to associate with each component the movement of a particular ion across the plasmalemma. This has been done successfully in squid axon (Hodgkin, Huxley, and Katz 1952) where it has been shown that the movement of Na^+ ions into the axon causes the initial rise in membrane potential, and a later movement of K^+ ions outwards causes the recovery with an overshoot past the resting level.

Osterhout (1934, 1936, 1944, 1947) and Osterhout and Hill (1935) have obtained records of action potentials in *Nitella flexilis* which show two components, but the first component was very short (0·5 sec) and the slow component had a greater magnitude than that measured by this author. Measurements of action potentials in *Nitella opaca* and *Nitella mucronata* made by Umrath (1932*a*, 1932*b*, 1953, 1954) also suggest two components. Records obtained by Oda (1956) on *Chara braunii* and by Sibaoka (1958) on *Nitella flexilis* show two components clearly. On the other hand, the action potential in *Nitella flexilis* recorded by Cole and Curtis (1938) does not show two components.

For *Nitella* grown in White's solution and in tap water, the magnitude of the action potential measured in these culture media is always less than the resting potential, and consequently the vacuole potential does not become positive. However, some measurements of action potentials (not described in this paper) which have been made on cells in White's solution 10–30 times the strength of the culture solution show some overshoot of the zero potential. Fujita and Mizuguchi (1955) have reported overshooting by 20 mV of the zero potential level in *Nitella*, and Oda (1956) observed overshoots of 50 mV in *Chara braunii*, but in the latter case, no details are given of the concentrations of culture solution and bathing solutions.

V. Acknowledgments

The author thanks Professor A. L. McAulay and Dr. B. I. H. Scott for their advice and criticism during the course of the experimental work and the preparation of this paper. Mr. D. E. Millwood designed and constructed the electrometers, without which this work would not have been possible.

VI. References

Cole, K. S., and Curtis, H. J. (1938).—Electric impedance of *Nitella* during activity. *J. Gen. Physiol.* **22**: 37–64.

Fujita, M., and Mizuguchi, K. (1955).—Generation of action potential in *Nitella*. *Bull. Electrotech. Lab.* **19**: 58–69.

Hill, S. E. (1941).—The relation of streaming and action potentials in *Nitella*. *Biol. Bull., Wood's Hole* **81**: 296.

Hill, S. E., and Osterhout, W. J. V. (1934).—Nature of the action current in *Nitella*. II. Special cases. *J. Gen. Physiol.* **18**: 377–83.

Hill, S. E., and Osterhout, W. J. V. (1938).—Nature of the action currents in *Nitella*. IV. Production of quick action currents by exposure to NaCl. *J. Gen. Physiol.* **22**: 91–106.

Hodgkin, A. L., Huxley, A. F., and Katz, B. (1952).—Measurement of current-voltage relations in the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**: 424–48.

MacRobbie, E. A. C., and Dainty, J. (1958).—Ion transport in *Nitellopsis obtusa*. *J. Gen. Physiol.* **42**: 335–54.

Oda, K. (1956).—Resting and action potentials in *Chara braunii*. *Sci. Rep. Tohoku Univ.* (4)**22**: 167–74.

Osterhout, W. J. V. (1927).—Some aspects of bioelectric phenomena. *J. Gen. Physiol.* **11**: 83–99.

Osterhout, W. J. V. (1931).—Physiological studies of single plant cells. *Biol. Rev.* **6**: 369–411.

Osterhout, W. J. V. (1934).—Nature of the action current in *Nitella*. I. General considerations. *J. Gen. Physiol.* **18**: 215–27.

Osterhout, W. J. V. (1936).—Electrical phenomena in large plant cells. *Physiol. Rev.* **16**: 216–37.

Osterhout, W. J. V. (1944).—Nature of the action current in *Nitella.* V. Partial response and the all-or-none law. *J. Gen. Physiol.* **27**: 61–8

Osterhout, W. J. V. (1947).—Nature of the action current in *Nitella.* VI. Simple and complex action patterns. *J. Gen. Physiol.* **30**: 47–59.

Osterhout, W. J. V., and Hill, S. E. (1935).—Nature of the action current in *Nitella.* III. Some additional features. *J. Gen. Physiol.* **18**: 499–514.

Sibaoka, T. (1958).—Conduction of action potential in the plant cell. *Trans. Bose Res. Ins.* **23**: 43–56.

Umrath, K. (1930).—Untersuchungen über Plasma und Plasmaströmung an Characeen. IV. Potentialmessungen an *Nitella mucronata* mit besonderer Berücksichtigung der Erregungserscheinungen. *Protoplasma* **9**: 576–97.

Umrath, K. (1932*a*).—Die Bildung von Plasmalemma (Plasmahaut) bei *Nitella mucronata.* *Protoplasma* **16**: 173–88.

Umrath, K. (1932*b*).—Der Erregungsvorgang bei *Nitella mucronata.* *Protoplasma* **18**: 258–300.

Umrath, K. (1934).—Der Einfluss der Temperatur auf das elektrische Potential, den Aktionsstrom und die Protoplasmaströmung bei *Nitella mucronata.* *Protoplasma* **21**: 329–34.

Umrath, K. (1953).—Über Aktionsstrom und Stillstand der Protoplasmaströmung bei *Nitella opaca.* *Protoplasma* **42**: 77–82.

Umrath, K. (1954).—Über die elektrische Polarisierbarkeit von *Nitella mucronata* und *Nitella opaca.* *Protoplasma* **43**: 237–52.

Walker, N. A. (1955).—Microelectrode experiments on *Nitella.* *Aust. J. Biol. Sci.* **8**: 476–89.

White, P. R. (1943).—"A Handbook of Plant Tissue Culture." (The Jacques Cattell Press: Pennsylvania.)

NODULATION STUDIES IN LEGUMES

III. THE EFFECTS OF DELAYING INOCULATION ON THE SEEDLING SYMBIOSIS OF BARREL MEDIC, MEDICAGO TRIBULOIDES DESR.

By P. J. Dart* and J. S. Pate*

[*Manuscript received June* 16, 1959]

Summary

The technique of delayed inoculation with effective, ineffective, or mixtures of effective + ineffective nodule bacteria was used to study seedling symbiosis of barrel medic, *M. tribuloides*, in sand culture.

Nodule production over the first 6 weeks of growth was stimulated in all bacterial series by inoculation delays of from 5 to 25 days. Maximum nodules were placed on primary roots by delays of 14–19 days, on lateral roots by delays of 25 days.

Following delayed inoculation, nodule initiation commenced on existing root-hair-invested portions of the root system. The highest nodule on the primary root usually developed 1–2 cm behind the root tip and more than two lateral primordia below the lowest lateral visible at inoculation.

Nodule frequencies, determined in each delay treatment for numbered laterals and for spaces between laterals on the primary root, were used to describe the "infection potential" of the root system. "Infection centres" were apparently distributed in a regular fashion down the primary root. With long inoculation delays, laterals developed clumps of closely packed nodules exhibiting nodule intensities (nodules/cm) 10–20 times those realized by inoculation at sowing. Results suggested that only a very small fraction of the infection potential was exploited under any inoculation regime.

Serological and visual typing of nodules in delay treatments using mixed inocula showed the ineffective partner competed much more successfully for positions on the primary root than might be expected from a sharing of the infection potential in proportion to the nodulating capacities of the respective strains in single culture.

There was abundant evidence that existing nodules restricted subsequent root growth and nodulation of host seedlings. These restrictive influences were cumulative and characteristic for the invading strain(s). Results were discussed with reference to possible exchange reactions between host plant and nodule tissues.

I. Introduction

The effects of delaying inoculation on the nodulation of agar cultures of red clover were described by Nutman (1949) in his series of investigations on the physiology of nodule development in legumes. Delays in inoculation with an effective *Rhizobium* were found to reduce the time lag between inoculation and the appearance of nodules on the root, and also led to much greater rates of nodule production than could be obtained by inoculation at sowing. Nodule production was not increased if severe nitrogen hunger conditions had developed in seedlings prior to inoculation. No permanent stimulus to infection rate was obtained by delaying inoculation with an ineffective *Rhizobium*.

---

* Botany School, University of Sydney, and Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., University of Sydney.

The results of the above experiments, together with others on the relationship between lateral root and nodule formation (Nutman 1948) and the effect of excising nodule apices on subsequent root and nodule development (Nutman 1952), were used to support a physiological hypothesis of host–plant interaction in nodule formation. This hypothesis, expanded by Nutman in later publications (Nutman 1956, 1958), proposed that identical meristematic foci were utilized in lateral root and nodule formation and that existing meristematic centres on the root produced inhibitory substances limiting further infection of the root system.

The study of barrel medic detailed in this paper is relevant to several of the issues mentioned above. However, the technique of delayed inoculation was used here primarily for other reasons, namely, to determine the precise site of infection on the root, to test whether competitive abilities of bacterial strains vary with time of inoculation of the seedling root system, and to describe the "infection potential" of different regions of the root. Sand culture was selected for these and other experiments so that the full implications of delayed inoculation could be examined over the complete symbiotic cycle.

II. Materials and Methods

(*a*) *Plant Culture and Inoculation Procedure*

Sand culture of barrel medic (*Medicago tribuloides* Desr., strain 173) was carried out under natural illumination in a glass-house in Sydney in May–August, 1957. Enamelled iron pots of 7 in. diameter and 4 l. capacity were filled with acid-washed Nepean R. sand and steam-sterilized prior to sowing. Seeds, refrigerated for 12 hr at 0°C to promote even germination, were surface-sterilized with mercuric chloride and sown 20 seeds to a pot. Each pot was watered to field capacity with distilled water and 300 ml of a standard nitrogen-free culture solution made up as follows: 0·8 g $CaSO_4$, 0·4 g K_2HPO_4, 0·2 g $MgSO_4.7H_2O$, iron as chelate (5 mg Fe/l), 1 ml of trace-element solution per litre of distilled water. The trace-element solution consisted of 2·86 g H_3BO_3, 1·81 g $MnCl_2.4H_2O$, 0·11 g $ZnCl_2$, 0·05 g $CuCl_2.2H_2O$, 0·025 g $Na_2MoO_4.2H_2O$ per litre distilled water. Sand cultures watered with culture solution showed a reaction of pH 6·7–7·0.

For the first 10 days after sowing, pots were covered with transparent polythene sheeting to reduce the chance of airborne contamination by foreign nodule bacteria and to prevent seedling injury through excessive drying out of pots.

Two serologically dissimilar strains of nodule bacteria were obtained from the University of Sydney School of Agriculture Collection and used as inoculants. One, *Rhizobium* SU41·237, was ineffective (I), the other, *Rhizobium* SU277·1, was fully effective (E) on the host variety. In experiment 1, a preliminary study of the biology of the two strains, 40 pots of medics were inoculated with I bacteria and another 40 pots with E bacteria to form two series from which periodic samples were taken to study seedling symbiosis following a normal inoculation at sowing.

In experiment 2 the effects of delaying inoculation were studied in three bacterial series:

(1) I 1–10, where sets of five pots of seedlings were inoculated with ineffective bacteria at 10 selected times in seedling growth.

(2) E 1–10, a similar series using effective bacteria.

(3) E+I 1–10, as above but using a mixed inoculum of effective plus ineffective bacteria at each inoculation time.

The inoculation schedule for the 10 treatments of each series is given in Table 1. Bacteria were applied to all treatments at a rating of more than 10^6 bacteria per pot giving an immediate rhizosphere density assumed to be well in excess of that required for maximum nodulation (see Purchase and Nutman 1957).

TABLE 1

SCHEDULE FOR THE DELAYED INOCULATION OF MEDICAGO TRIBULOIDES SEEDLINGS

Treatment* Number	Time of Inoculation		Seedling Condition at Inoculation†		
	Days from Sowing	Days from Germination	Primary Root Length (cm)	Lateral Roots per Plant‡	Top Development
1	3	0	0·2	0	
2	5	2	1·2	0	
3	7	4	2·3	0	Cotyledons expanded
4	9	6	2·9	0	
5	12	9	5·7	0·4	
6	14	11	7·1	2·0	Spade leaf expanded
7	16	13	9·0	7·8	
8	19	16	11·0	14·2	
9	22	19	12·6	17·3	First trifoliate leaf expanded
10	25	22	14·5	22·1	

* Each treatment comprised an E, I, and E + I series inoculated respectively with effective, ineffective, and a mixture of effective + ineffective nodule bacteria (E = strain SU277·1, I = strain SU41·237). Five replicate pots, each of 10 plants, were established for each treatment of a series.

† Seedling development determined from a sample of 40 uninoculated plants.

‡ Only those lateral roots projecting 1 mm or more beyond the primary root surface were counted.

At each inoculation time 40 plants were extracted from pots of similarly aged uninoculated seedlings to determine host–plant condition at inoculation and to check that seedlings being inoculated had remained unnodulated (see Table 1). Eventual nodule distribution patterns, determined at harvest, also showed that airborne contamination had not produced nodules prior to artificial inoculation. There was no evidence in the experiment of contamination of the I series by E bacteria or vice versa although this did occur sporadically in long-term experiments using other legumes and strain combinations (see Pate 1958*b*). The success in maintaining controls sterile and preventing later contaminations was attributed to the precautions taken to exclude dust from the glass-house, to the separation of inoculated pots from uninoculated controls, and to the use of sterile equipment when applying bacteria or water to the seedlings.

Post-emergence thinning reduced seedlings to 10 per pot. Pots were watered daily to field capacity with distilled water. and 100 ml of nitrogen-free culture solution was added weekly to each pot.

(*b*) *Harvesting and Recordings from Samples*

(i) *Experiment* 1.—Samples of 40 plants were taken from both E and I series at 2- or 3-day intervals in seedling development. The following quantities were measured—nodule numbers on primary and lateral roots, average size (fresh weight), and colour (white, red, or green) of nodules on the primary root (see Pate (1958*a*) for further details of nodule colour classes, sampling procedure, etc.).

(ii) *Experiment* 2.—A single harvest was made 39 days from sowing. Samples of 30 plants were taken from the five replicate pots of each of the 30 treatments. All quantities listed for experiment 1 were recorded. Nodule distribution maps were constructed for each treatment where the frequency of occurrence of nodules was recorded for each numbered lateral root and for the numbered spaces between laterals on the primary root. The average distance below the hypocotyl junction to the first nodule on the primary root was determined for each treatment.

In the E + I series, primary root nodules were classed on a visual basis as either E or I using criteria obtained from experiment 1 (see Section III). Serological typing, using the method of Vincent (1941), was used to check this morphological separation of nodule types. Visual typing was not possible for the populations of young nodules on lateral roots of the E + I series.

III. RESULTS

(*a*) *The Biology of the Effective and Ineffective Bacterial Associations with Barrel Medic* (*Experiment* 1)

The following paragraphs summarize the principal features of the two types of symbiosis over the first 30 days of seedling growth.

Infection of the seedling axis produced the first visible nodule in either series at about 10 days from sowing. The final intensity of nodulation on the primary root was similar for both strains.

Nodule pigmentation on the primary root occurred in both nodule types over the 15–20-day period of seedling growth. All E nodules remained red and in active growth throughout the period of the experiment. In the I series nodule haemoglobin was rapidly decomposed, giving nodules formed by this strain a maximum pigmented life of 5 days. Coincident with haemoglobin disappearance in the I nodule a pronounced collapse of the meristem was observed.

Rapid and extensive infection of lateral roots occurred in the I series resulting in the formation at 26 days of approximately three times as many lateral root nodules as on E-nodulated plants. There is an interesting coincidence between the appearance of size and colour differences in primary root nodules in the responses of the two strains and the establishment of contrasting infection patterns on lateral roots (see also experiment 2).

In summary, the strains exhibit typical effective and ineffective behaviour although the I strain is not totally ineffective since some fixation return might be expected from the small ephemeral core of haemoglobin-pigmented tissue. The visual characteristics selected from this experiment as criteria for distinguishing E and I nodules in experiment 2 are as follows:

I Nodules.—Crater-shaped apex with aborted meristem, brown or green contents, hemispherical outline.

E Nodules.—Active meristem, red pigmentation, size at least twice that of contemporary ineffective nodules.

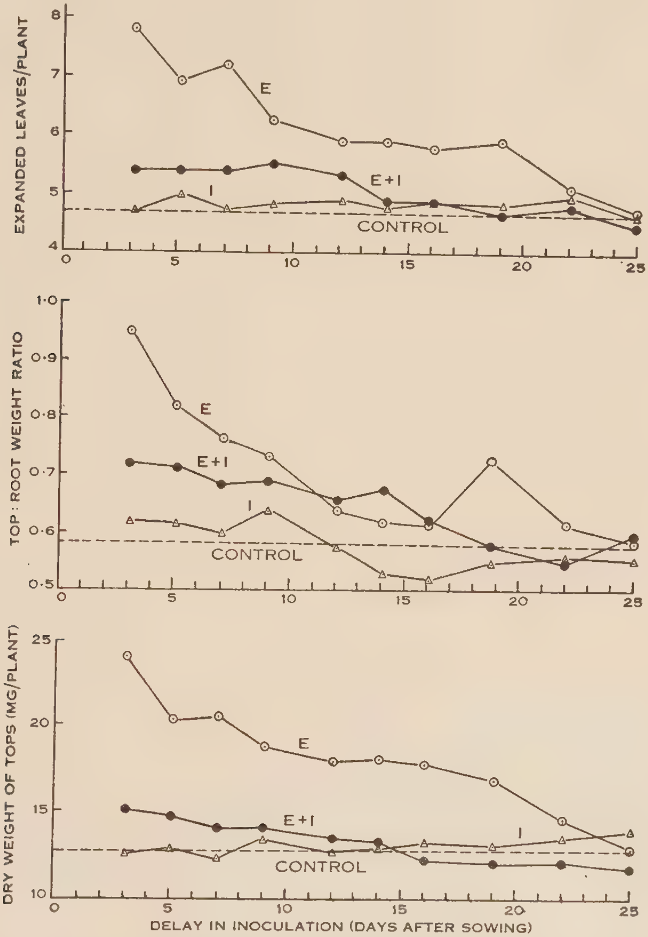


Fig. 1.—Experiment 2: Delayed inoculation of *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective+ineffective (E+I) bacteria. Effects of delayed inoculation on various features of host plant development at 39-day harvest. Each recording is a mean value derived from a sample of 30 plants.

(*b*) *The Effects of Delaying Inoculation on Seedling Symbiosis of Barrel Medic (Experiment* 2)

Effects of delay in inoculation on host development and nodulation are listed for the three bacterial series in the following subsections.

(i) *Dry Matter Production in Plant Tops, Leaf Development, and Top : Root Weight Ratio* (*Fig.* 1).—The benefits of early nodulation by E bacteria, and to a lesser extent by E + I bacteria, are seen in greater values in all of the above quantities compared with growth of uninoculated controls or I-nodulated plants. The longer E nodules were present on a root system, the greater the difference from control growth. Quantities recorded for the E + I series at different delay periods approximate more closely to those of the I series than the E series. This is consistent with the observation evaluated later that with all delay times involving mixed inocula a greater proportion of nodules were formed by the ineffective strain. The presence of I nodules on roots clearly detracts from the full benefit of E nodulation.

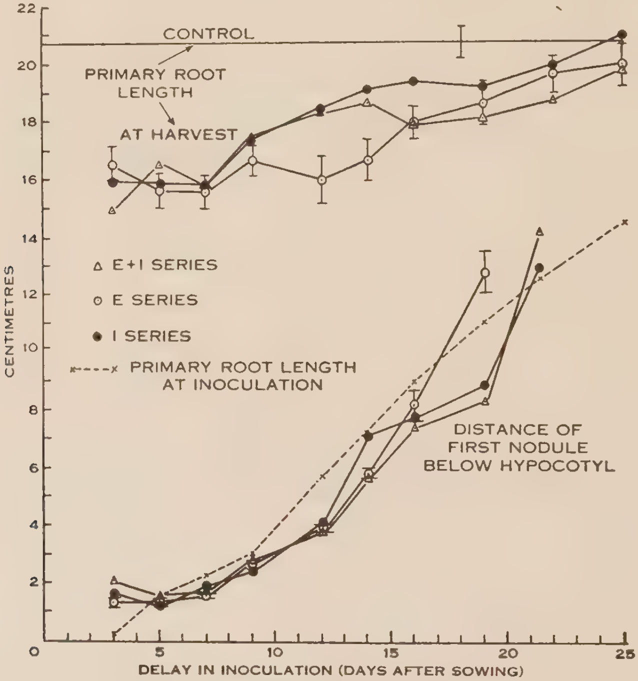


Fig. 2.—Experiment 2: Delayed inoculation of *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective+ineffective (E+I) bacteria. Primary root extension in the three bacterial series and in control uninoculated plants at 39-day harvest. In the lower half of the figure distances below the hypocotyl to the highest nodule are compared with primary root length at inoculation. Experimental variability is indicated by standard errors of means of treatments of the E series.

(ii) *Primary Root Extension and Lateral Root Production* (*Figs.* 2 *and* 3).—In all series there is a marked reduction in both primary root growth and lateral formation when performances of inoculated plants are compared with uninoculated root growth. The maximum reduction in these quantities is obtained by inoculation at germination (3 days after sowing) and shows a root approximately 5 cm shorter and bearing 15 fewer laterals than similarly-aged uninoculated plants. At greater delay intervals in the three series the degree of reduction is proportional to the length of time that nodule bacteria have had access to the primary root (see Figs. 2 and 3).

(iii) *Nodule Production* (*Fig.* 4).—The overall effect of delaying inoculation in all three bacterial series is to promote the production of substantially greater numbers of nodules at harvest than in the plants inoculated at germination. A two- or three-fold increase in the nodule numbers was obtained in the period of the experiment with the longest delay treatment used.

Primary root infection.—With increasing delay interval, primary root nodules increase slightly but significantly in all series, with maxima attained by withholding the inoculum for 14–19 days (primary root 7–11 cm at inoculation). Longer delay led to the production of fewer nodules as sites on the upper part of the root became closed to infection and as seedlings entered a severe nitrogen-hunger condition.

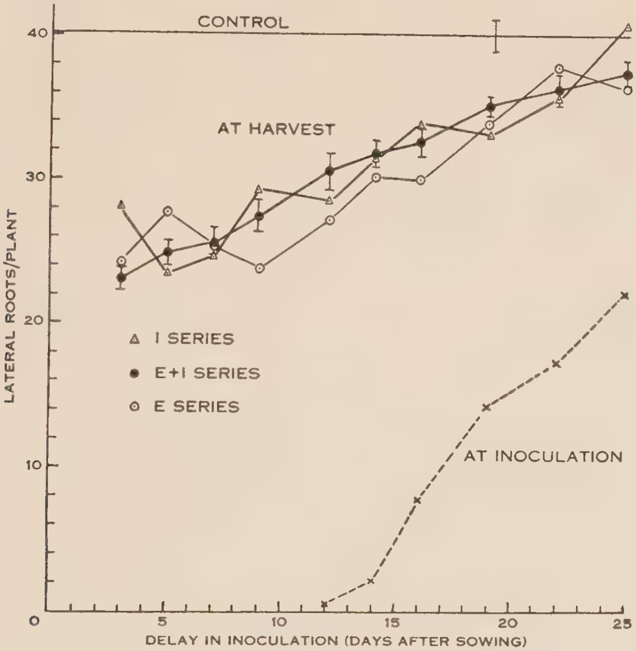


Fig. 3.—Experiment 2: Delayed inoculation in *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective+ineffective (E+I) bacteria. Lateral root production in the three bacterial series and in control uninoculated plants at 39-day harvest. Laterals visible on plants at inoculation are shown in the lower half of the diagram. Experimental variability is recorded as standard errors of means of treatment of the E+I series.

Lateral root infection.—The more substantial increase in lateral root nodulation with increasing delay in inoculation shows maxima in the three series of some 60 nodules per plant with the longest delay period of 25 days. It is probable that if even longer delay periods had been used, nitrogen starvation of the root would have restricted nodulation in a similar manner to that described for red clover by Nutman (1949), and reproduced in delayed-inoculation studies of other legumes (Pate and Dart, unpublished data).

The curves of lateral root nodulation against inoculation delay in Figure 4 show two response phases. Firstly, over the delay range increasing from 3 to 16 days there is a gradual increase in nodule numbers, with evidence of a differential response between strains. In this phase the I series nodulation is consistently about double that of the E series. The E + I response yields intermediate nodule numbers, with a closer approximation to the E response with short delays but tending to approach I values with longer delays. This last result can be correlated with the observation (described later) that the percentage of I nodules developing from mixed inocula increases the longer inoculation is delayed (see Section IV).

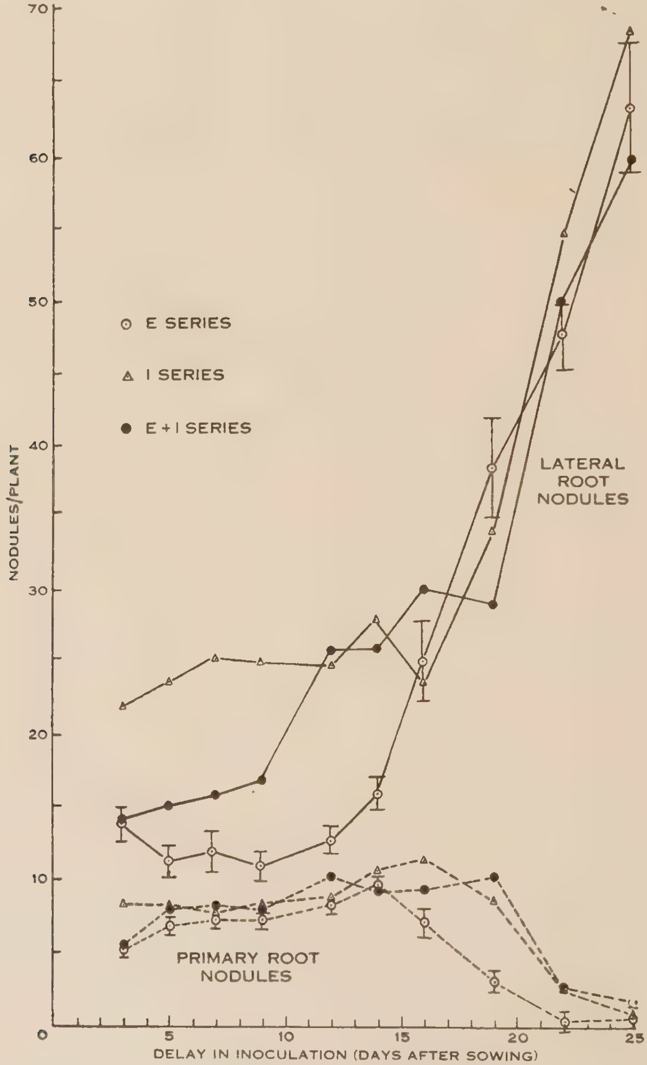


Fig. 4.—Experiment 2: Delayed inoculation in *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective + ineffective (E+I) bacteria. Nodule production at 39-day harvest. Standard errors of means of the treatments of the E series are included as an indication of experimental variability.

The second phase of response (16–25 days delay) witnesses a marked increase in lateral nodulation with delay. In this phase no strain differences were established during the time of the experiment.

(iv) *Nodule Distribution at Different Delay Treatments* (*Fig.* 5 *and Plate* 1).—Four delay intervals of 3, 12, 16, and 22 days are selected to illustrate nodule frequencies in the three series in relation to their spatial occurrence on the root system. Nodule distributions are expressed in the form of histograms where the frequency of occurrence of nodules is recorded for each lateral root numbered from the hypocotyl and for each numbered space between laterals on the primary root (see Fig. 5). The three series show remarkably similar distribution patterns in response to increasing delay in inoculation:

1. Inoculation at sowing or with a delay of up to 9 days allows successful bacterial exploitation of any space between laterals on the primary root. There is a slight preference in all series for infection to occur in positions on the primary root between and above the first three laterals below the hypocotyl, i.e. a slight tendency towards crown nodulation. Similarly, there is a tendency for the upper laterals to be more abundantly nodulated, although here it must be borne in mind that older and longer laterals are likely to carry larger populations of nodules.
2. With delay periods of more than 12 days upper regions of the primary root become closed to infection and in consequence the main nodule-bearing zone becomes placed further and further down the root as inoculation is progressively delayed. There is a concomitant increase in lateral root nodulation particularly on those laterals opposite the upper nodule-free portions of the primary root.
3. In the E + I series, primary root nodule populations show an increasing proportion of I nodules with delay in inoculation.
4. Distal portions of roots develop "clumps" of nodules where inoculation is delayed for more than 16 days. This "clumping" effect is illustrated in the photographs of individual root systems (see Plate 1). The histograms of Figure 5 also depict the overall increase in nodule intensity on the infected portion of the primary root and on secondary roots with increasing inoculation delay.

(v) *The Site of Infection on the Primary Root as Determined by Delayed Inoculation.*—The lower portion of Figure 2 compares average primary root length at inoculation with the average distances down the primary root to the first nodule with different inoculation treatments. In the delay range up to 5 days, a root grows some 1 or 2 cm after inoculation before producing a nodule. With delays of more than 7 days but less than 20 days portions of the primary root present at inoculation appear open to infection, with the highest nodule usually developing in a region 0·5–2 cm shorter than the primary root length at inoculation.

A second description of the site of infection can be made by comparing secondary root development at inoculation with the eventual spacing of primary root nodules between numbered secondary roots on the seedling axis. In the frequency

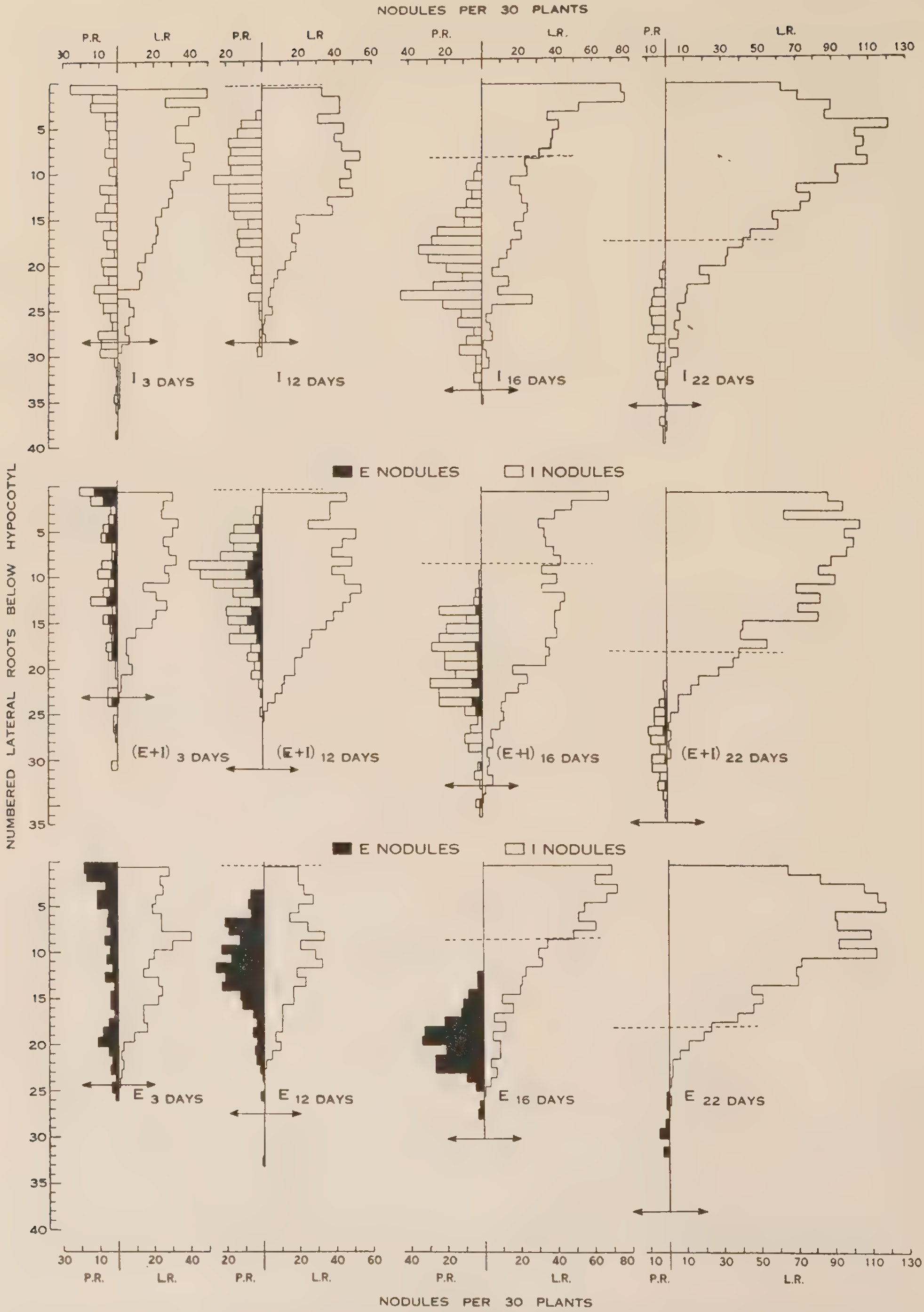


Fig. 5.—Experiment 2: Delayed inoculation in *M. tribuloides* using effective (E), ineffective (I), and mixtures of effective+ineffective (E+I) bacteria. Histograms of nodule distribution are recorded for the three bacterial series at delay treatments of 3, 12, 16, and 22 days. Nodule

[*For continuation see opposite page.*]

distribution patterns of Figure 5 there is clearly a space difference representing some 2–10 secondary roots between average secondary root number at inoculation (broken line across top of histogram) and the first space recorded as being likely to be occupied by a nodule.

IV. Discussion

The present study of delayed inoculation on barrel medic provides information on the site of infection and utilization of the nodule-forming potential of the root under different inoculation regimes. In addition quantitative interactions between symbionts are evident which are particularly relevant to current theories on the nature, origin, and activity of the legume nodule.

(*a*) *The Site and Intensity of Nodulation*

Application of nodule bacteria at any time during primary root extension in barrel medic usually leads to infection of existing portions of the root. A root-hair-invested zone some 1–2 cm behind the root tip and more than two lateral primordia below the lowest lateral visible at inoculation is prescribed as the region where the highest nodule on the primary root is likely to develop. The nodule-distribution patterns following different delays in inoculation suggest that bacterial entrance in barrel medic is restricted to zones of the root showing healthy root hair development.

The site of infection following a very short inoculation delay reveals that the top few centimeters of root below the hypocotyl junction but above the first lateral root are normally unsuited to bacterial exploitation. Root hairs are absent or poorly developed in this region. The sparse nodulation of the base of primary roots following long delay in inoculation suggests that the nitrogen-hunger state of the seedling induced by prolonged absence of external or symbiotic sources of nitrogen renders roots fit for only occasional nodulation.

Nodule-distribution maps for primary roots depict nodulation as occurring in any space between laterals, the spaces exploited most fully depending on the infection circumstances. In Table 2 the maximum nodulation frequency per space between laterals on the primary root is recorded for each series from pooled observations of the 10 delay treatments. 26 nodules per 30 plants per space between adjacent laterals is obtained as the mean of the maximum frequencies recorded in any series. The uniform figures recorded for the top 30 spaces between laterals on the primary root suggest the existence of regularly distributed infection foci down the primary root (see also Nutman 1952, 1958). In this connection the distribution maps of Figure 5 stress that the marked increase in nodule intensity (nodules/cm) on the lower primary root with increasing delay in inoculation is clearly effected through a fuller use of the maximum nodulating potential of adjacent spaces between laterals rather than abnormally high nodule frequencies in any one or more of these spaces.

frequencies for the 30 plants of a treatment sample are recorded for each lateral root numbered from the hypocotyl and for the numbered spaces between laterals on the primary root. Effective and ineffective nodules are distinguished in the primary root frequency distributions of the E+I series. – – – – Average laterals on root at inoculation. ⟷ Average laterals on root at harvest.

Tetraploid cells in the root cortex are implicated as predetermined nodule-forming initials in the studies of Wipf and Cooper (1940), and Fujita and Mitsuishi (1953). On such an assumption the present study would favour that initials would be present on a root far in excess of the normal demands of nodulation. Indeed a

TABLE 2

DELAYED INOCULATION OF MEDICAGO TRIBULOIDES

Maximum nodule frequencies recorded for the numbered spaces between laterals on the primary root

Space Number*	Maximum Nodule Frequency (nodules per space per 30 plants)†			
	E Series	I Series	E + I Series	Combined Series‡
1	19	26	22	26
2	20	19	16	20
3	22	20	13	22
4	15	15	12	15
5	18	11	15	18
6	17	13	19	19
7	21	18	17	21
8	19	17	23	23
9	13	18	40	40
10	23	17	34	34
11	18	26	27	27
12	26	25	16	26
13	21	18	27	27
14	23	31	29	31
15	16	17	19	19
16	25	13	15	25
17	15	24	29	29
18	21	27	26	27
19	32	34	22	34
20	32	29	26	32
21	28	19	14	28
22	26	25	28	28
23	26	26	29	29
24	10	44	24	44
25	14	21	15	21
26	9	16	24	24
27	10	15	11	15
28	5	20	22	22
29	7	16	27	27
30	5	12	22	22
Average for 30 spaces	19 ± 1·3	21 ± 1·3	22 ± 1·2	26 ± 1·2

* Spaces numbered from hypocotyl down root, e.g. space 1 is region above 1st lateral, space 9 between 8th and 9th laterals, etc.

† Frequencies selected from pooled observations of the 10 treatments of a series.

‡ Maximum frequencies for any series.

condition approaching a full utilization of nodule foci in a particular region of the root might be encountered only in special infection conditions, e.g. the high nodule intensities on distal roots following excessive delay in inoculation. Furthermore, full exploitation of all infection foci in a root segment may require the use of an abundantly nodulating bacterial strain, which might be pictured as being particularly efficient in the fulfilment of the sequences leading to the location and stimulation of a nodule initial in the root cortex. It is quite clear that only a very small fraction of the infection potential of the seedling root system is exploited under any particular inoculation regime.

(*b*) *Strain Competition for Access to Roots and Delayed Inoculation*

Multiple infection of a host legume is a well-known natural phenomenon and has also been reproduced in culture by inoculating legumes with two or more invasive bacteria (e.g. see work of Nicol and Thornton 1941; Erdman and Means 1951; Vincent and Waters 1953). It is generally conceded that the host plant exerts the selective influence which may lead to a differential uptake of bacteria in root nodulation. Differently timed applications of contrasting bacterial strains have been used to study whether antecedence by one strain confers immunity against subsequent invasion by a second strain (e.g. results of Lohnis 1930; Chen 1941; Virtanen and Linkola 1947; Burton and Allen 1949). It is clear from these studies that both the root growth pattern of the host plant and the number and type of existing nodules influence the subsequent course of infection.

Results here add further to present knowledge by demonstrating that bacterial acceptance for nodule formation from mixed inocula varies with the time of inoculation of the host legume root system. Table 3 shows that the proportion of ineffective nodules in the E + I barrel medic series increases four-fold as inoculation is delayed from 3 to 25 days. The effect bears little relationship to the performances of the respective strains in pure culture in response to delay. This can be seen by comparing the observed values for the nodulation of the two strains in mixed culture with comparable values for the nodulation of the strains in pure culture. The marked and significant divergence of observed from expected values with the longer delay periods reveals that the ineffective partner appears to compete more successfully for lower positions on the primary root than might be expected from a sharing of the nodule-forming potential in proportion to the nodulation capacities of the strains in pure culture. This effect may reflect a differential response of the two strains to a changing biochemical condition in the host root following continued root expansion in nitrogen-deficient conditions. A full evaluation is not possible until it is known whether discrimination between strains takes place at the root hair or subsequent to bacterial invasion.

(*c*) *Nodule–Host Plant Interactions*

In the establishment of any legume the first-formed symbiotic organs and considerable portions of root and shoot system develop at the expense of cotyledon reserves. Therefore it is not surprising to find in a small-seeded legume like barrel medic that early host plant development is greatly influenced by the time and type of nodulation.

(i) *An Increase over Control Uninoculated Growth in Leaf and Dry Matter Production with Effective Symbiosis.*—The extent of this normal response to effective symbiosis obviously depends on the number and duration of activity of nodules on the seedling root. The higher top : root weight ratio in E as opposed to I symbiosis indicates that the early products of fixation are directed towards top development (see similar results obtained for other annual legumes (Pate 1958*a*, 1958*b*)). It is noted below that nodulation actually restricts root growth in the seedling legume: this effect will also tend to favour elevation of the top : root ratio. With continued nitrogen fixation one might expect an ultimate benefit to both root growth and further nodulation. Such a stimulus was not observed within the time limits of this experiment but was obtained in other delayed-inoculation experiments.

Table 3

DELAYED INOCULATION OF MEDICAGO TRIBULOIDES

Comparison of primary root nodulation using effective (E), ineffective (I), and a mixture of effective + ineffective (E+I) bacteria

Treatment Number*	% E Nodules on Primary Root (observed)† with 99% Confidence Limits (E+I Series)	% E Nodules on Primary Root (expected)‡	Treatment Number*	% E Nodules on Primary Root (observed)† with 99% Confidence Limits (E+I Series)	% E Nodules on Primary Root (expected)‡
1	45·4 ± 5·51	39·3	6	16·2 ± 2·48	47·5
2	31·8 ± 3·03	45·5	7	11·4 ± 1·65	38·4
3	34·4 ± 2·76	48·0	8	13·1 ± 2·48	26·3
4	31·0 ± 2·20	46·1	9	11·0 ± 2·76	14·1
5	24·8 ± 2·48	48·3	10	—	17·8

* See Table 1 for inoculation schedule.

† The means of the 30 individual plant percentage E values in each E+I treatment. E and I nodules were distinguished on a visual and serological basis for all treatments except the small nodules produced following the longest delay interval of 25 days (treatment 10).

‡ E nodules in pure culture ÷ (I nodules in pure culture + E nodules in pure culture) expressed as a percentage. This expected value assumes that nodulation in mixed culture is proportional to the performances of the respective strains in pure culture.

(ii) *A Reduction below Control Uninoculated Growth in Primary Root Extension and Lateral Production associated with the Presence of Nodules on the Primary Root.*—The degree of reduction in these quantities bears an inverse relationship to inoculation delay. No differences in reaction between the three bacterial series were observed within the time limits of the experiment, suggesting that nodule-induced suppression of root growth in the seedling legume is not dependent on continued nitrogen fixation in the nodule (Figs. 2 and 3).

(*d*) *Nodule–Nodule Interactions*

Two features of the nodulation responses to delayed inoculation suggest an interaction between nodules. The first of these is the apparent suppression of lateral root nodulation by existing primary root nodules. The intensity of this effect is related to the position of the primary root nodule zone, and in the short delay treatments a differential strain response is evident where an E nodule on a primary root appears to exercise a more restrictive influence than an I nodule. The second feature is the higher nodule numbers and intensities associated with long delays in inoculation. Both bacterial strains exhibited these responses although greater stimulation of nodule numbers with delayed inoculation occurred in the effectively nodulated series.

The following assumptions are introduced to interpret the observed responses of barrel medic to delayed inoculation:

(1) Nodule initiation on a particular segment of root is dependent on a combination of the following conditions in the host plant: (i) the presence of young and healthy root hairs; (ii) the presence beneath the root hair zone of suitable infection centres in the cortex for bacterial exploitation; (iii) a suitable biochemical–physiological condition in the root for nodule formation. Any of these prerequisites may limit nodulation. Thus, the patterns of infection with different inoculation delays expose the essentiality of condition (i); situations are suggested above (see Section IV(*a*)) where requisite (ii) might limit nodulation; condition (iii) might restrict nodulation in nitrogen-deficient tissues, or, as is suggested in paragraphs below, if existing nodules were to alter the physiology of the root in such a way as to inhibit subsequent infection and nodule development.

(2) An existing nodule appears to exercise a localized and cumulative influence tending to restrict further nodulation, lateral development, and primary root extension of the seedling.

(3) The restrictive influences exerted by a nodule increase as the nodule ages. Stimulation of nodule numbers or infection rates by delayed inoculation can then be interpreted as follows: the later inoculation is delayed the larger the area of root-hair-invested root that is presented to the bacteria at inoculation, and hence the more nodules that can develop initially on the various segments of infectable root before the earlier-formed of these nodules impose restrictions on further infection.

(4) In addition to the interpretation presented in (3) the clumping effect produced by excessive delay in inoculation implies that resistance of the host plant to infection is lowered as the root system expands in nitrogen-deficient media in the absence of nodule bacteria. This enables an extraordinary number of foci to be utilized on root portions susceptible to invasion at inoculation. The infection thread–nodule initiation sequences of roots are now being studied to determine the efficiency of bacterial utilization of the infection potential of the root with different delay treatments.

(5) The restrictive influence of a maturing nodule is dependent on its size and growth activity. In the strain pair studied here, E and I nodules appeared to exercise quantitatively similar effects for the first 2 or 3 weeks of their growth. Thus the three bacterial series show almost identical nodulation responses on primary roots and similar restrictive influences on primary root growth and lateral initiation (Figs. 2, 3, and 4). Differences in potency of inhibitory influence developed when nodules were 2–3 weeks old, coincident with the appearance of size, colour, and meristem differences in the two nodule types. The greater restrictive influence of the mature E primary root nodule is reflected in the fact that nodulation of lateral roots following inoculation at germination or with moderate delays is consistently less extensive in the E series than in the E + I or I series (see Fig. 4). Nutman (1949) described much greater differences between nodule production responses to delayed inoculation using an effective and ineffective strain on red clover. The ineffective association he used might be designated as abundantly nodulating, where the small nodules produced would exert only a meagre and transient restrictive influence on further nodulation of the root.

Two hypotheses may be advanced as alternative interpretations of the mechanism whereby existing nodules might affect host plant development and nodulation. The first, a *nutritional* hypothesis assumes that certain nutrient substances are utilized in both root and nodule development and that an existing nodule (or lateral) intercepts and sequesters these substances in their passage down the root, thereby restricting development distal to the developing nodule (or lateral). It should be noted that this hypothesis is compatible with anatomical objections raised by Allen and Allen (1958) against Nutman's (1949) hypothesis of identical meristematic foci on the root being used in lateral and nodule formation. In this connection it is pictured that nutrient factors delivered to the infected region would be shared in either the fulfilment of pericyclic stimulation for lateral production or in the successful exploitation of cortical initials by bacteria in nodule formation.

The second hypothesis assumes that nodules, *per se*, produce inhibitors whose cumulative effect is to arrest primary root growth and attendant lateral or nodule formation. This *inhibitor* hypothesis is essentially that introduced by Nutman (1949, 1958) as a possible mechanism for the regulation of nodule and lateral root numbers on the host legume. As stated above the hypothesis need not be coupled with Nutman's hypothesis of an origin of laterals and nodules from identical foci in the root. Pate (1958*c*) has shown that legume nodules contain large amounts of several growth substances. A backward diffusion of inhibitory concentrations of such substances from the nodule might be held responsible for the nodule–root growth interactions described here and in Nutman's studies on red clover.

Until further information is available on the biochemistry and physiology of exchange reactions between nodule and root either hypothesis remains acceptable. In future experiments it is hoped to approach the problem by combining delayed inoculation with timed applications of nutrient substances to top or root to test whether the arrested root growth and nodulation associated with existing nodules can be wholly or partially alleviated by the supply of extra, possibly limiting, nutrients to the seedling. As a sideline to these studies it may be possible to specify an ideal inoculation–fertilizer treatment promoting maximum symbiosis over the life cycle of the association.

V. ACKNOWLEDGMENTS

We are indebted to Professor J. M. Vincent, Faculty of Agriculture, University of Sydney, for his useful criticisms and interest in this work and for his permission to use glass-house space in the Agriculture School. We would also like to thank Mrs. L. M. Waters for her supply of the bacterial strains used in this study, and Mr. J. B. Bloomfield for his advice on the use of serological techniques.

VI. REFERENCES

ALLEN, E. K., and ALLEN, O. N. (1958).—Biological aspects of symbiotic nitrogen fixation. In "Encyclopedia of Plant Physiology". Vol. 8. pp. 48–118. (Springer-Verlag: Berlin.)

BURTON, J. C., and ALLEN, O. N. (1949).—Inoculation of crimson clover (*Trifolium incarnatum* L.) with mixtures of rhizobia strains. *Proc. Soil Sci. Soc. Amer.* **14**: 191–5.

CHEN, H. K. (1941).—The limited number of nodules produced on legumes by different strains of *Rhizobium*. *J. Agric. Sci.* **31**: 479–87.

ERDMAN, L. W., and MEANS, U. M. (1951).—Single strains and a mixture of *Rhizobium trifolii* for inoculating true clovers. *Proc. Soil Sci. Soc. Amer.* **16**: 199–200.

FUJITA, T., and MITSUISHI, S. (1953).—Cytological studies on the root nodules of peas. *Proc. Crop. Sci. Soc. Japan* **22**: 97–8.

LOHNIS, M. P. (1930).—Investigations upon the ineffectiveness of root-nodules on Leguminosae. *Zbl. Bakt.* (*Abt.* 2). **80**: 342–68.

NICOL, H., and THORNTON, H. G. (1941).—Competition between related strains of nodule bacteria and its influence on infection of the legume host. *Proc. Roy. Soc.* B **130**: 32–59.

NUTMAN, P. S. (1948).—Physiological studies on nodule formation. I. The relation between nodulation and lateral root formation in red clover. *Ann. Bot., Lond.* **12**: 81–96.

NUTMAN, P. S. (1949).—Physiological studies on nodule formation. II. The influence of delayed inoculation on the rate of nodulation in red clover. *Ann. Bot., Lond.* **13**: 261–83.

NUTMAN, P. S. (1952).—Studies on the physiology of nodule formation. Experiments on the excision of root tips and nodules. *Ann. Bot., Lond.* **16**: 80–101.

NUTMAN, P. S. (1956).—The influence of legume in root-nodule symbiosis. A comparative study of host determinants and functions. *Biol. Rev.* **31**: 109–51.

NUTMAN, P. S. (1958).—The physiology of nodule formation. In "Nutrition of the Legumes". Proc. Univ. Nottingham 5th Easter School in Agricultural Science. pp. 87–107. (Butterworths Scientific Publications: London.)

PATE, J. S. (1958*a*).—Nodulation studies in legumes. I. The synchronization of host and symbiotic development in the field pea, *Pisum arvense* L. *Aust. J. Biol. Sci.* **11**: 366–81.

PATE, J. S. (1958*b*).—Nodulation studies in legumes. II. The influence of various environmental factors on symbiotic expression in the vetch (*Vicia sativa* L.) and other legumes. *Aust. J. Biol. Sci.* **11**: 496–515.

PATE, J. S. (1958*c*).—Studies of growth substances of legume nodules using paper chromatography. *Aust. J. Biol. Sci.* **11**: 516–28.

PURCHASE, H. F., and NUTMAN, P. S. (1957).—Studies on the physiology of nodule formation. VI. The influence of bacterial numbers in the rhizosphere on nodule initiation. *Ann. Bot., Lond.* **21**: 439–54.

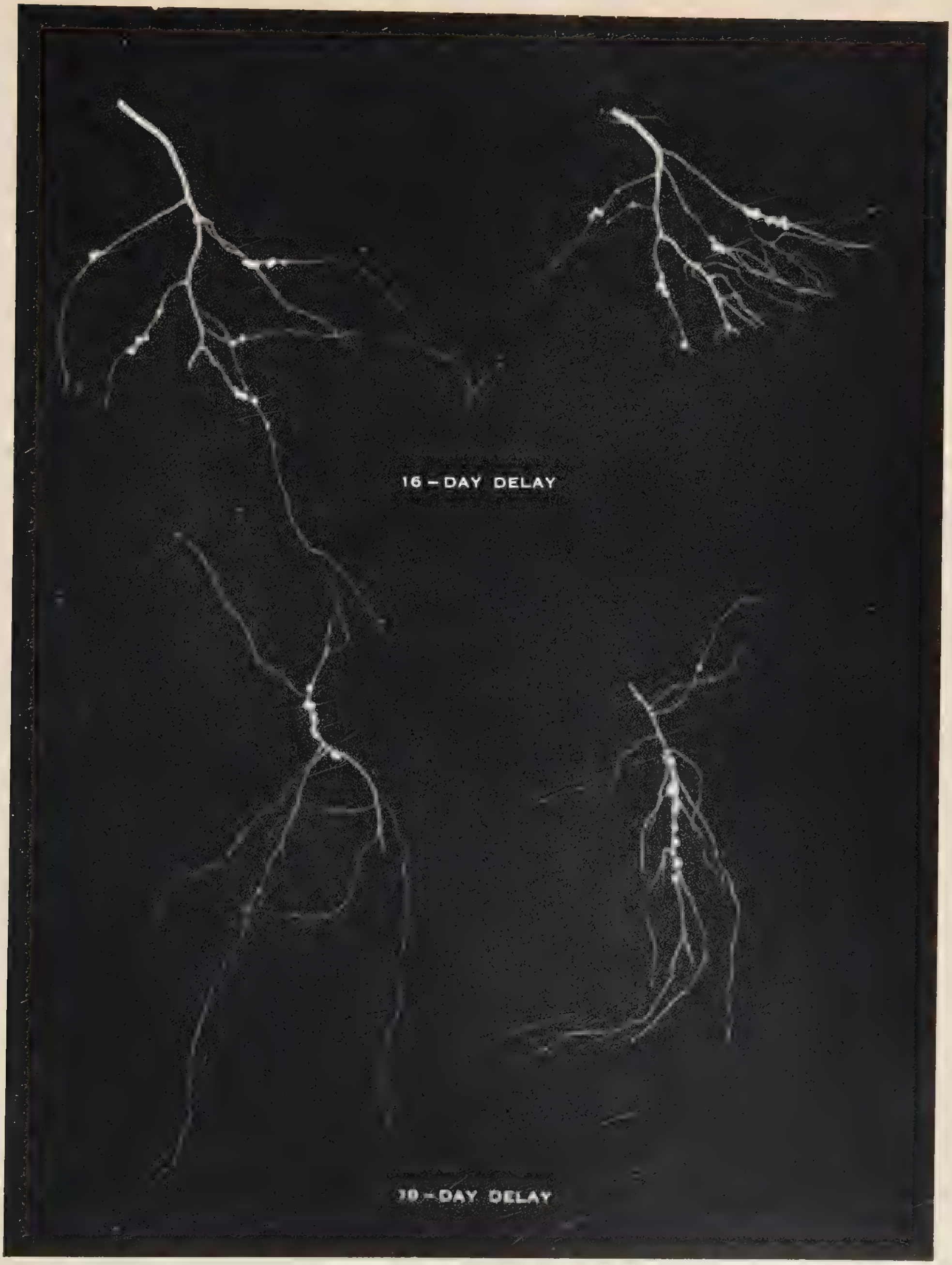
VINCENT, J. M. (1941).—Serological studies of the root-nodule bacteria. I. Strains of *Rhizobium meliloti*. *Proc. Linn. Soc. N.S.W.* **66**: 145–54.

VINCENT, J. M., and WATERS, L. M. (1953).—The influence of host on competition amongst clover root nodule bacteria. *J. Gen. Microbiol.* **9**: 357–70.

VIRTANEN, A. I., and LINKOLA, H. (1947).—Competition of *Rhizobium* strains in nodule formation. *Antonie van Leeuwenhoek* **12**: 65–77.

WIPF, L., and COOPER, D. C. (1940).—Somatic doubling of chromosomes and nodular infection in certain Leguminosae. *Amer. J. Bot.* **27**: 821–4.

NODULATION STUDIES IN LEGUMES. III



Portions of root systems of 39-day-old barrel medic (*M. tribuloides*) seedlings showing the characteristic clumping of nodules on distal root segments following long delays in inoculation. Upper two photographs: infected laterals after a 16-day delay in inoculation; lower two photographs: infected zone of primary roots after a 19-day delay in inoculation. Invading *Rhizobium* SU277·1. ×1.

CHEMICAL CHANGES DURING GROWTH AND STORAGE OF THE AVOCADO FRUIT

By J. B. Davenport* and S. C. Ellis*

[*Manuscript received May* 13, 1959]

Summary

Changes in the petrol-soluble, 80 per cent. ethanol-soluble, and insoluble fractions of the mesocarp of the avocado fruit were followed during 6 months development on the tree and during storage at 20°C of the fully grown fruit. Changes in triglyceride fatty acids, lipid phosphorus, insoluble phosphorus, sugars, protein, and soluble nitrogen were also studied.

During development there was a large increase in the triglyceride oil content with a concomitant fall in water content. Monoene acids were principally synthesized, the saturated and polyene acids being laid down at an early stage in the development of the fruit. A considerable proportion of polar homolipids was shown to be present in the fruit.

The C_7 sugars may play an important role in the metabolism of the fruit and lipid material probably contributes to respiration. Phospholipid content was high in the young fruit but fell in the first month to a steady value. Synthesis of protein occurred during storage and the changes taking place during storage varied according to the stage of development of the fruit.

I. Introduction

Studies on the avocado fruit were undertaken primarily to elucidate the pattern of fatty acid synthesis during growth and storage, and to relate this to other chemical changes in the mesocarp tissue. Early studies of the chemical constitution of the mesocarp of the fruit of *Persea gratissima* (*P. americana*) were reviewed by Wardlaw (1937). Interesting features of the chemical composition are the very high fat content (up to 30 per cent. of the fresh tissue) and the presence of a heptitol, perseitol (Maquenne 1890), and an aldoheptose, *manno*heptulose (La Forge 1916). Investigation of the composition of the triglyceride oil (Asenjo and Goyco 1942; Alvarez *et al.* 1949) has revealed the presence of myristic, palmitic, stearic, arachidic, palmitoleic, oleic, docosenoic, linoleic, and linolenic acids. The work described here shows that the diene and triene acids are laid down principally in the very young fruit, and subsequent fatty acid synthesis is mainly of monoene acids. The fruit contains a considerable amount of homolipids more polar than the triglycerides and the data suggested that the C_7 sugars may play an important part in the metabolism.

II. Methods

(*a*) *Sampling*

Fruit of the Fuerte variety was obtained from a farm at Redland Bay, S. Qld., at the beginning of each month from January to June 1957. It was flown to Sydney on the day of picking, and analysis commenced the next day. The fruit

---

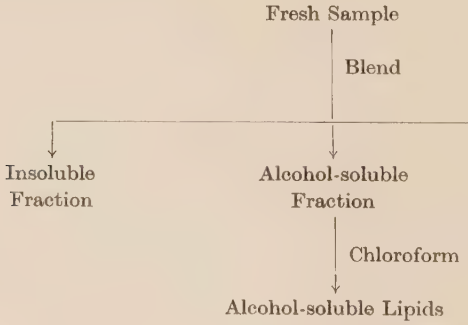
* Division of Food Preservation and Transport, C.S.I.R.O., Homebush, N.S.W.

was picked from four trees, the fourth unfortunately yielding no more fruit after the April picking. Details of these pickings are set out in Table 1. The fruit was fully grown by April though May is considered the month of commercial maturity. The fruit in May was slightly larger than in June, probably due to selective picking of the larger fruit. In January, February, and March the fruits were weighed on arrival and divided into two samples, A and B, each sample containing the same number of fruits from each tree and with a similar range of individual fruit weights. In April, May, and June, the batch was divided into four samples A, B, C, and D on the same basis. A and B were analysed immediately, while C and D were analysed after storage for a period at 20°C, during which respiration was measured and the fruit became ripe (i.e. soft and edible).

For analysis, a longitudinal segment was cut from each fruit and peeled. A thin longitudinal slice of constant thickness was cut from each segment, halved, and alternate distal and stem end pieces combined in the sample, which usually weighed 25–30 g. Comparable duplicate samples were used for moisture determinations.

(*b*) *Fractionation*

The fresh sample was blended with ethanol (sufficient to give a final concentration of 80 per cent. aqueous ethanol) and light petroleum (b.p. 60–70°C) at 1°C. After blending, the insoluble residue was filtered off, washed thoroughly with 80 per cent. aqueous ethanol and light petroleum, dried, and weighed. This was a light-coloured friable powder. The two phases of the filtrate were separated. The petrol-soluble material was weighed after evaporation of the petrol, and the ethanol-soluble fraction was diluted to 250 ml. An aliquot of this alcoholic solution was evaporated to dryness and weighed to determine alcohol-soluble solids. The dry residue was extracted with chloroform and the chloroform extract was weighed after evaporation of the solvent. The fractionation procedure is summarized in the following diagram:



(*c*) *Analysis*

Nitrogen and phosphorus were determined on the insoluble fraction and reducing sugars and nitrogen on the alcohol-soluble fraction. The petrol-soluble fraction was saponified and acid value, iodine value, and ultraviolet absorption after alkaline isomerization determined on the isolated acids. Phosphorus content and ultraviolet absorption after alkaline isomerization were determined on the alcohol-soluble lipids. The following analytical procedures were used: nitrogen by the microKjeldahl procedure of McKenzie and Wallace (1954); lipid phosphorus by the method of Allen (1940) as modified by Rhodes (1955); insoluble phosphorus by the method of Ging (1956); reducing sugars by the ferricyanide method of Ting (1956); acid value by the official method of the American Oil Chemists' Society (1946*a*); iodine value by the official method of the American Oil Chemists' Society (1946*b*); alkaline isomerization with 1·0M potassium *tert.*-butoxide for 2 hr at 100°C (Davenport, Birch, and Ryan 1956), the percentages of the various fatty acids being calculated using the following equations:

$$\text{Percentage triene acid} = 1\cdot314k_{268}.$$
$$\text{Percentage diene acid} = 1\cdot013k_{233}-0\cdot734k_{268}.$$

Table 1

DETAILS OF SAMPLING OF AVOCADO FRUITS

Date of Picking	No. of Trees	No. of Fruit from Each Tree	Average Weight (g)	Range in Weight (g)
8. i.57	4	8	99	58–147
8. ii.57	4	8	195	144–264
6.iii.57	4	8	288	181–379
3.iv.57	4	16	296	213–444
1. v.57	3	8	309	226–422
3.vi.57	3	16	299	169–417

The monoene acid and saturated acid content was calculated in the usual way from the iodine value and percentages of diene and triene acids. The residue from the alcohol-soluble fraction after extraction with chloroform was de-ionized with a mixture of "Amberlite" resins (IR–4B.OH and IR–120.H) and the residual sugars were chromatographed on paper with ethyl acetate–*n*-propanol–water (7 : 2 : 1 by volume) as solvent. The papers were sprayed with alkaline silver nitrate (Trevelyan, Procter, and Harrison 1950) and with the orcinol–trichloroacetic acid–acetic acid reagent of Klevstrand and Nordal (1950). The latter gave a blue colour with *manno*heptulose, a pale green with fructose, and did not react with the other sugars present.

III. Results

(*a*) *Segregation in the Fractionation Procedure*

It could be assumed on the basis of known solubilities that the petrol-soluble fraction would contain the triglycerides and unsaponifiable matter, the alcohol-soluble fraction reducing sugars, amino acids, and phospholipids, and the insoluble fraction polysaccharides and proteins. None of the petrol-soluble fractions contained phosphorus, hence no phospholipid went into the petrol phase. A surprising feature was the high proportion (30 per cent. or greater) of the alcohol-soluble fraction which was soluble in chloroform. Phosphorus analyses indicated that the alcohol-soluble lipids contained approximately 10 per cent. phospholipid and, consistent with this, approximately 10 per cent. was insoluble in acetone. A preliminary examination of the acetone-soluble alcohol-soluble lipids by absorption chromatography revealed that it consisted of a complex mixture of lipids containing only carbon, hydrogen, and oxygen. Further chemical work on the characterization of these lipids is in progress and will be reported separately. They appear to be lipids of a type not previously described.

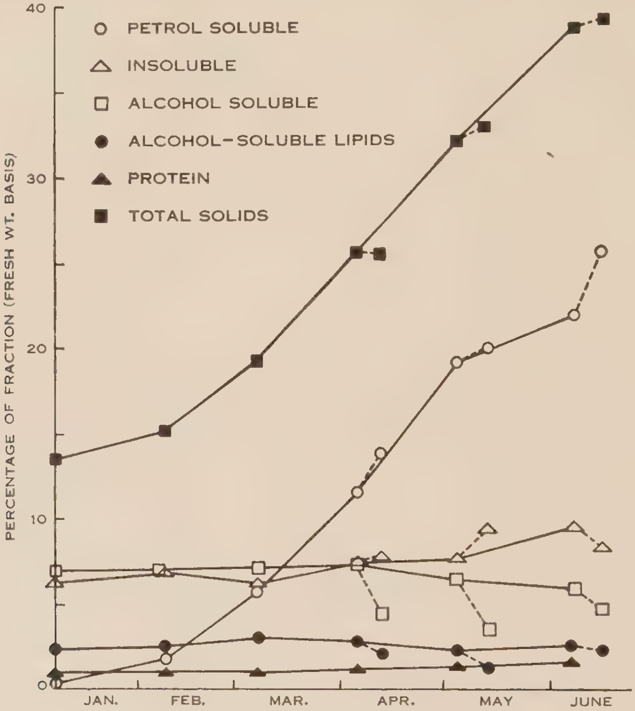


Fig. 1.—Percentage changes, on a fresh weight basis, of the various fractions of the mesocarp during development (solid lines) and storage (broken lines). The standard errors of the means, with nine degrees of freedom, were as follows: petrol-soluble fraction, 0·48; insoluble fraction, 0·31; alcohol-soluble fraction, 0·25; alcohol-soluble lipids, 0·11; protein, 0·04; total solids, 0·49.

Also, the triglycerides were confined wholly to the petrol phase, none or very little appearing in the alcohol-soluble lipids fraction. Chromatographic examination of the combined unsaponifiable matter from the petrol-soluble fractions revealed that it consisted principally of hydrocarbon and sterol fractions and contained negligible amounts of the substances present in the alcohol-soluble lipids fraction.

Hence it appears that the fractionation achieved good segregation of the various lipid types. A considerable amount of phosphorus was found in the insoluble fraction and initially it was thought that this might be due to the phospholipid associated with the protein. Hence part of the insoluble fraction of sample A (June picking) was refluxed with an ethanol–diethyl ether (3 : 1 v/v) mixture, and filtered. The filtrate was taken to dryness, yielding 5·2 per cent. of lipid which contained no phosphorus and which was almost completely soluble in light petroleum. This appears to be lipid more firmly bound to protein or polysaccharide or both than that in either the petrol-soluble or the alcohol–soluble lipids fractions. It was found that all the insoluble phosphorus could be extracted in the cold with 1·5N perchloric acid, and that the extract showed a fairly broad absorption maximum at 256 mμ, indicating the presence of nucleotides. However, the insoluble residue of sample A (June picking) contained 6·8 mg-atoms of phosphorus and only 0·025 m-moles of nucleotide (assuming an extinction coefficient (ϵ) of 10,000). Hence most of the insoluble phosphorus is present in other forms.

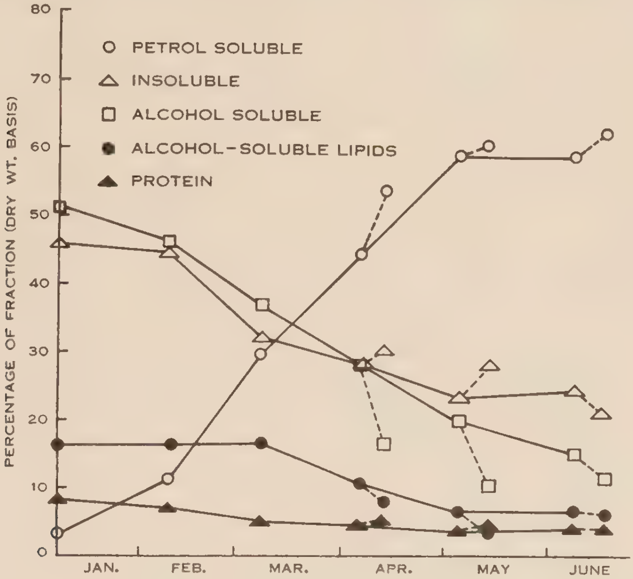


Fig. 2.—Percentage changes, on a dry weight basis, of the various fractions of the mesocarp during development (solid lines) and storage (broken lines).

(b) *Changes during Development of the Fruit on the Tree*

Percentage changes in the insoluble, alcohol-soluble, petrol-soluble, and alcohol-soluble lipids fractions have been plotted on a fresh weight basis in Figure 1, and on a dry weight basis in Figure 2. Percentage protein has been calculated by multiplying the percentage insoluble nitrogen by 6·25. The analytical values for nitrogen, phosphorus, and reducing sugars of the various fractions are plotted on a fresh weight basis in Figure 3 and on a dry weight basis in Figure 4. Changes in the percentages of the various types of fatty acids in the petrol-soluble fraction are plotted on a dry weight basis in Figure 5, and the results of the alkaline isomerization of the alcohol-soluble lipids fractions listed in Table 2. Each point on the graphs is the average of values for the duplicate fruit samples.

In Figure 1 and 2 it may be seen that marked synthesis of triglyceride oil took place mainly over a 3-month period (from February to April). In May the apparent increase in oil on a fresh weight basis was merely due to a loss of water from the fruit. Reference to Figure 5 indicates that it was principally monoene acids which were being synthesized, saturated and diene acids increasing only slightly, and triene acids remaining virtually constant.

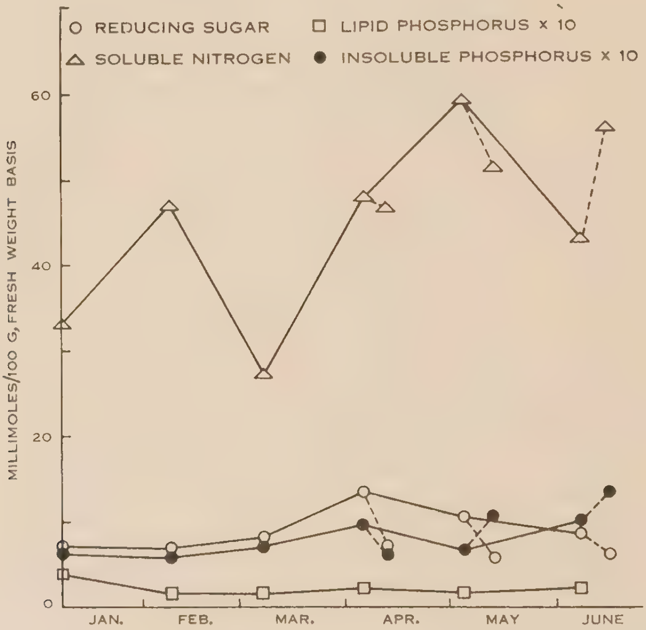


Fig. 3.—Changes on a fresh weight basis of various chemical entities in the mesocarp during development (solid lines) and storage (broken lines). The standard errors, with nine degrees of freedom, were as follows: reducing sugar, 0·84; soluble nitrogen (alcohol-soluble fraction), 2·3; lipid phosphorus, 0·03; insoluble phosphorus, 0·05.

The concentrations of insoluble, alcohol-soluble, alcohol-soluble lipids fractions and protein on a fresh weight basis did not change markedly, their apparent decrease on a dry weight basis being due to a proportional increase in oil content. In May, however, there was a definite fall in the alcohol-soluble fraction which was due mainly to a fall in reducing sugar concentration and some loss of soluble nitrogenous compounds. The phospholipid content fell sharply in January and thereafter remained fairly constant. The isomerization analysis of alcohol-soluble lipids showed a sharp rise in conjugatable diene and a fall in conjugatable triene during January and both then remained fairly constant.

From January to May there was a parallel between changes in reducing sugar and in insoluble phosphorus. In May, however, the reducing sugars continued to fall, whereas the insoluble phosphorus increased. Paper chromatography of the sugars revealed the presence in each monthly sample of sucrose, perseitol, *manno*-heptulose, and an unknown sugar, possibly a disaccharide, with an R_F near maltose (hereafter referred to as the unknown disaccharide). With the solvent system used, *manno*heptulose and glucose were not separated, but a comparison of the intensity of the reactions with alkaline silver nitrate and orcinol–trichloroacetic acid indicated that glucose was present. Paper chromatography did not reveal any marked variation in the proportion of the various sugars throughout the period studied except in May, when the perseitol concentration was higher than in the other months.

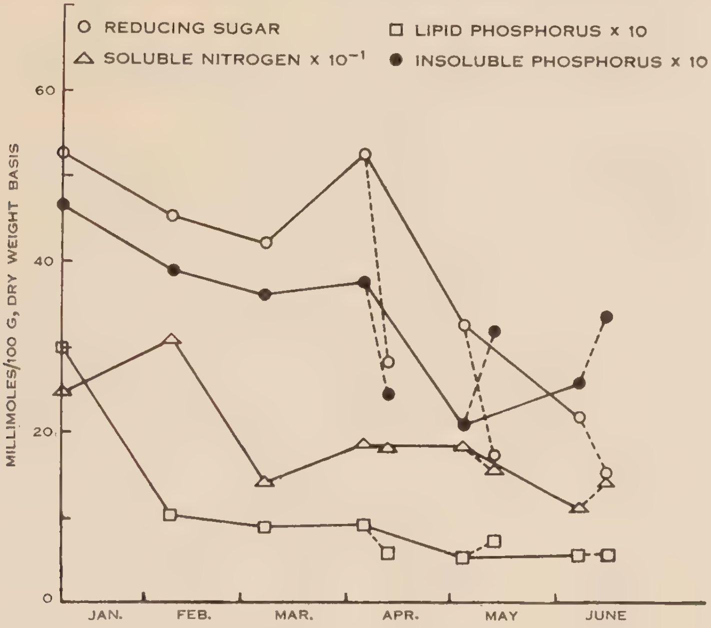


Fig. 4.—Changes on a dry weight basis of various chemical entities in the mesocarp during development (solid lines) and storage (broken lines).

(c) *Changes during Storage*

The respiration of the three stored samples was measured. The April sample did not pass through a well-defined climacteric and the fruit had only partly softened when it was analysed. In May and June the respiration exhibited the usual well-defined climacteric and the analysed samples were typically soft and edible. In Figures 1–5, the changes during storage are represented by the broken lines.

It is apparent from the figures that the changes during storage were dependent upon the stage of development of the fruit on the tree. There was only a slight amount of fat synthesized during storage, and marked falls in the alcohol-soluble fraction, due principally to a loss of sugar and, to a lesser extent, of lipids. Paper chromatography of the sugars showed that in April the perseitol almost disappeared, the *manno*heptulose decreased slightly, fructose and sucrose increased, and the unknown disaccharide increased. In May the perseitol and *manno*heptulose almost disappeared, the fructose and the unknown disaccharide increased markedly, and the sucrose did not change appreciably. In June both perseitol and *manno*heptulose practically disappeared, fructose increased markedly, while sucrose and the unknown disaccharide both increased. In April and May there was a slight synthesis of protein and a somewhat greater increase of insoluble material. Synthesis of

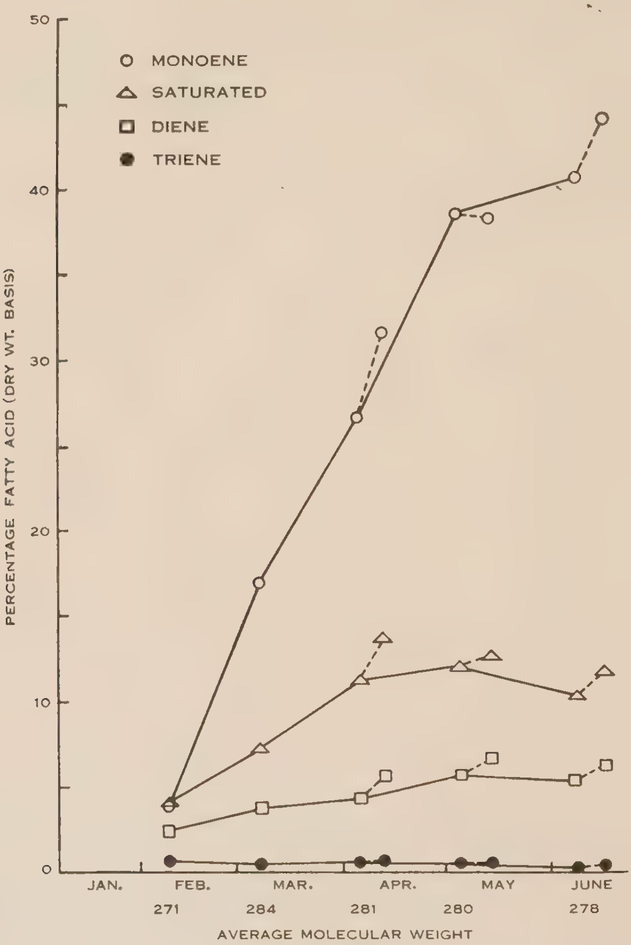


Fig. 5.—Percentage changes, on a dry weight basis, of the various classes of petrol-soluble fatty acids during development (solid lines) and storage (broken lines). The standard errors, with seven degrees of freedom, were as follows: saturated, 0·65; monoene, 1·56; diene, 0·14; triene, 0·13.

protein during storage has also been noted, and correlated with high energy phosphate and the climacteric rise of the fruit by Rowan, Pratt, and Robertson (1958). In June, however, the protein did not change and the insoluble fraction decreased markedly. The insoluble phosphorus increased in April but decreased in May and June. There were no striking changes in lipid phosphorus or soluble nitrogen except for a marked increase of the latter in June.

TABLE 2

ALKALI ISOMERIZATION OF ALCOHOL-SOLUBLE LIPIDS*

Results are expressed as extinctions ($E^{1\%}_{1\,\mathrm{cm}}$) at the wavelengths for conjugated diene (233 mμ) and conjugated triene (268 mμ) developed after isomerization

Wavelength	Jan.	Feb.	Mar.	April		May		June	
				Before Storage	After Storage	Before Storage	After Storage	Before Storage	After Storage
233 mμ	131	229	230	237	236	237	237	237	260
268 mμ	86	46	31	31	33	43	51	41	34

* Preliminary work on the acetone-soluble portion of the alcohol-soluble lipids fraction indicates that this conjugation does not develop necessarily from unsaturated fatty acids, although some may arise from unsaturated fatty acids in the phospholipids.

IV. DISCUSSION

A microscopic examination of the avocado mesocarp shows that the fat droplets accumulate in the vacuole of the cells. In the ripe fruit the vacuole presents a striking appearance, being packed with oil droplets. The fall in the water content of the fruit during development may be due to a displacement of water from the vacuoles by the accumulating oil droplets. During the phase of rapid fat synthesis, monoene acids were almost exclusively synthesized. The Fuerte avocado oil is unusual in that it contains about 6 per cent. of palmitoleic acid and about 3 per cent. of docosenoic acid, as well as about 55 per cent. of oleic acid (Alvarez *et al.* 1949). The average molecular weight of the fatty acids was constant throughout, and possibly all three monoene acids were being synthesized. Stumpf and Barber (1957) have demonstrated that mitochondria isolated from avocados incorporate labelled acetate into oleic and palmitic acids under anaerobic conditions, most activity appearing in the former. This result is consistent with the predominant synthesis of monoene acids by the whole fruit.

The early rise in conjugatable diene and fall in conjugatable triene in the alcohol-soluble lipids appeared to be associated with a fall in the phospholipid content of that fraction, and lend support to the concept of an early establishment of the pattern of polyunsaturated acids in the fruit, which then remains fairly constant throughout subsequent development and storage. During storage in May and June there were small but significant changes in the composition of the oil. The monoene acid content fell while that of the saturated, diene, and triene acids tended to rise, suggesting that the fatty acids are involved, to some extent, in metabolic changes during storage. The alcohol-soluble lipids, as well as the reducing sugars, appeared to contribute to the respiration during storage, and this may account for the low R.Q. observed by Biale (1946).

During storage the C_7 sugars almost disappeared, leaving the more usual pattern of C_6 sugars. A simple explanation is that the C_7 sugars were not synthesized in the fruit, but translocated to it from other parts of the tree; hence, when the fruit was picked, they were metabolized with no replacement. Nordal and Benson (1954) have isolated *manno*heptulose and its monophosphate from avocado leaves. They were unable to isolate it from several varieties of the fruit, and this may have been due to the fact that ripe fruit was used. They concluded that heptulose metabolism in the avocado was sluggish and did not play an important role in the metabolism of the plant, but the present results indicate that it is an active substrate for respiration in the fruit.

V. Acknowledgments

The authors are indebted to Dr. A. A. Benson for a gift of authentic samples of perseitol and *manno*heptulose, to Dr. J. F. Turner who performed the paper chromatography of the sugars, and to Drs. R. N. Robertson, F. E. Huelin, J. F. Turner, D. H. Turner, and K. S. Rowan for helpful discussion. We are also indebted to Mr. H. W. L. White, Orchardist, Redland Bay, Qld. for his helpful cooperation.

VI. References

Allen, R. J. L. (1940).—*Biochem. J.* **34**: 858.

Alvarez, J., Cattaneo, P., Grandolini, E., Karman, G., and Rigotti, J. P. (1949).—*An. Asoc. Quím. Argent.* **37**: 34. (*Chem. Abstr.* **43**: 9489 (1949).)

American Oil Chemists' Society (1946*a*).—Official and tentative methods. No. L 3a–55. Acid value of commercial fatty acids.

American Oil Chemists' Society (1946*b*).—Official and tentative methods. No. Cd 1–25. Iodine value of commercial fats and oils.

Asenjo, C. F., and Goyco, J. A. (1942).—*Oil & Soap* **19**: 129.

Biale, J. B. (1946).—*Amer. J. Bot.* **33**: 363.

Davenport, J. B., Birch, A. J., and Ryan, A. J. (1956).—*Chem. & Industr.* **1956**: 136.

Ging, N. S. (1956).—*Anal. Chem.* **28**: 1330.

Klevstrand, R., and Nordal, A. (1950).—*Acta Chem. Scand.* **4**: 1320.

La Forge, F. B. (1916).—*J. Biol. Chem.* **28**: 511.

Maquenne, M. (1890).—*Ann. Chim.* (*Phys.*) **19**: 5.

McKenzie, H. A., and Wallace, H. S. (1954).—*Aust. J. Chem.* **7**: 55.

Nordal, A., and Benson, A. A. (1954).—*J. Amer. Chem. Soc.* **76**: 5054.

Rhodes, D. N. (1955).—*Nature* **176**: 215.

Rowan, K. S., Pratt, H. K., and Robertson, R. N. (1958).—*Aust. J. Biol. Sci.* **2**: 329.

Stumpf, P. K., and Barber, G. A. (1957).—*J. Biol. Chem.* **227**: 407.

Ting, S. V. (1956).—*Agric. & Food Chem.* **4** : 263.

Trevelyan, W. E., Procter, D. P., and Harrison, J. S. (1950).—*Nature* **166**: 444.

Wardlaw, C. W. (1937).—*Trop. Agriculture, Trin.* **14**: 34.

HYPOTHYROIDISM IN THE SHEEP

By A. K. LASCELLES* and B. P. SETCHELL†

[*Manuscript received June* 17, 1959]

Summary

The effect of the oral administration of methyl thiouracil at three dose rates to pregnant ewes has been studied. Observations were made on foetal skeletal growth and maturity, foetal and maternal thyroid iodine, plasma-protein-bound iodine (PBI), cholesterol, phospholipid, total esterified fatty acids, and calcium. A retardation of osseous growth and a significantly greater retardation of osseous maturity was found. The thyroid iodine concentration in both ewes and foetuses and the maternal total thyroid iodine in all treated groups were decreased but the *foetal* total thyroid iodine was reduced only in the two highest dose rates. Significant reduction in the foetal PBI was found only with the two highest dose rates and no significant changes were found in the maternal PBI concentration. Increases in all lipid fractions and a decrease in calcium concentration were found in the foetal plasma in the treated groups.

I. INTRODUCTION

Hypothyroidism induced by various goitrogens is known to be associated with perinatal mortality in lambs. The disease was first reported in New Zealand by Gilruth (1901) who described hypertrophy in the thyroid glands in calves, lambs, and foals. Hopkirk *et al.* (1930) considered that the goitres were due to iodine deficiency. Sinclair and Andrews (1954) described the occurrence of congenital goitre in lambs when the pregnant ewes had been grazed on kale. Subsequently Wright and Sinclair (1958) presented evidence which suggested that the goitrogenic substance in kale belonged to the thiouracil group. Thiouracil is known to inhibit the organic binding of iodine as thyroglobulin (Vanderlaan and Vanderlaan 1947; Stanley and Astwood 1948; Berson and Yalow 1955). Flux *et al.* (1956) suggested that level of thiocyanate in the sera of animals fed clovers containing cyanogenetic glucosides was sufficient to produce goitre. Thiocyanate is known to be goitrogenic by inhibiting thyroidal accumulation of iodine (Astwood 1943; Rawson, Tannheimer, and Peacock 1944; Wolf *et al.* 1946; Barker, Lindberg, and Wald 1941; Brown-Grant and Gibson 1955).

Todd, Wharton, and Todd (1938) described the effect of thyroidectomy in the lamb, and noted defective development of the epiphyses and adjacent shafts. The present study was undertaken to examine the effects of hypothyroidism on ossification and growth in the foetal appendicular skeleton. Also biochemical observations on plasma-protein-bound iodine (PBI), lipid, calcium, and thyroid iodine concentrations were made.

---

* Department of Veterinary Medicine, University of Sydney.

† Nutrition Research Laboratory, Veterinary Research Station, Glenfield, N.S.W.

II. Materials and Methods

(a) *General*

Fifty Peppin-type Merino ewes were paddock-mated at Trangie Experimental Farm, N.S.W., between April 8 and 28, 1958. Twenty-four of those which did not return to service were transported to Sydney, arriving at the Veterinary School on May 25. The ewes were randomly divided into four groups of six ewes and were suitably identified. Following weighing, the 24 ewes were housed at random, three per pen. At each subsequent weekly weighing the ewes were re-randomized to the pens in order to prevent development of intra-pen correlations in the final results. The cardinal signs of each ewe were recorded daily and heparinized blood samples were collected at intervals of 5–6 weeks throughout pregnancy, the first sample being collected on May 27. The plasma was separated by centrifugation and stored at −20°C until analysed. Treatment of the ewes with methyl thiouracil commenced on June 3, three groups receiving respectively 0·5, 1·5, and 4·5 g of methyl thiouracil in 30 ml of water per day, by oesophageal tube. The ewes of the fourth group (i.e. controls) were given 30 ml of water. This dose range was based on the doses fed to young rams by Hall and Harvey (1952).

Soon after parturition, plasma samples were collected from both ewe and lamb. Each lamb was weighed and the body length measured by placing a piece of string along the mid-dorsal line from a line joining the medial canthi of the eyes to the tip of the tail (Dun 1955). The string was marked at the appropriate point and then compared with a fixed scale.

Post-mortem examination of each ewe and lamb was carried out. The thyroid glands were weighed. A small slice (approx. 0·5 g) was taken from each gland for total thyroid iodine estimations. The slice was weighed and placed in 10 ml of 12·5 per cent. formol–saline in a jar which was tightly stoppered.

Both dorsal and lateral radiographs of the left fore and hind limbs of each lamb were taken, using non-screen film (Ilford) at 50–55 kV, 30 mA, with 0·5 sec exposure and 36-in. anode film distance. By means of calipers the length of the tibial diaphysis was measured using the lateral radiograph, the proximal and distal landmarks taken being points midway between the anterior and posterior aspects of the bone. The degree of osseous maturity of lambs at birth was assessed by counting the number of appendicular epiphyseal and round bone centres of ossification, ignoring the lesser trochanter and the talus (Lascelles 1959).

(b) *Biochemical Methods*

PBI was determined by the method of Grossman and Grossman (1955) as modified by Setchell (1959). Ten simultaneous analyses on a single sample of plasma yielded a mean value of 4·51 μg PBI/100 ml with a standard error of the mean of ± 0·29. Eight analyses on the same plasma sample to which 5 μg of inorganic iodine/100 ml had been added immediately before drying and ashing gave the value of 9·35 μg/100 ml with a standard error of the mean of ±0·38. This indicates a recovery rate of 97 per cent., a satisfactorily high value.

Thyroid iodine was determined by digesting the weighed slice of thyroid gland in 5 ml ethanol and 10 ml of 1·8N KOH (Franklin 1931). During digestion, the volume was reduced by evaporation to about 5 ml to make the KOH approximately 4N. The volume was then made up to 50 ml in a volumetric flask with 4N Na_2CO_3. If necessary, further dilutions with 4N Na_2CO_3 were made; 1-ml portions of a suitable dilution were dried, incinerated, and the iodine determined by the above method for PBI. Dry weight of thyroids was taken as 20 per cent. of the fresh weight.

Plasma cholesterol was determined by the method of Pijoan and Walters (1937). Plasma total esterified fatty acids were determined by the method of Stern and Shapiro (1953). Phospholipids were determined in the extract for fatty acids, a 7-ml portion of which was evaporated to dryness in a microKjeldahl flask and digested with 1·2 ml 7N $HClO_4$. The inorganic phosphate was determined in the digest by the vanado-molybdate method of Simonsen *et al.* (1946).

Plasma calcium concentrations were determined by the spectrophotometric murexide method of Harper (1959). All readings were made on a Beckman spectrophotometer using 1-cm cells.

III. Results

(*a*) *Clinical Effects and Post-mortem Observations*

After a few weeks of treatment the ewes receiving the 1·5-g and the 4·5-g doses became noticeably lethargic. These animals rested for long periods and consumed less food than those in the other groups. Only two ewes from the 1·5-g group survived until parturition. The sheep that died showed characteristic symptoms, namely, progressive loss in weight, profound depression, and inappetance. The symptoms increased in severity until the ewe was unable to stand. Three of the ewes developed pneumonic symptoms in the terminal stages and areas of consolidation were found in the lungs at post-mortem examination.

All the lambs carried to full term were born alive but those in the treatment groups were weak and with few exceptions died soon after birth.

The mean length of the gestation period of each group was estimated by using the mid-range of the mating period as the date of conception. Since the groups were randomly formed this estimate is not biased, and a significant difference ($P < 0{\cdot}001$) is found between the length of the gestation period in the control and treated groups (cf. Sinclair and Andrews 1954).

The administration of the goitrogen did not affect the weight of the pregnant ewes surviving the gestation period. Although the body length of the lambs was significantly smaller in the treated groups ($P < 0{\cdot}05$) the difference in body weight just failed to be significant.

At autopsy, the most outstanding feature was the enlarged thyroid glands in those that had been treated with methyl thiouracil. However, the weight of the thyroid glands of the ewes in the 0·5- and 1·5-g groups was much greater than that of the only surviving ewe in the 4·5-g group. This particular gland had a

TABLE 1

DETAILS OF POST-MORTEM AND RADIOLOGICAL DETAILS OF EWES AND LAMBS TREATED WITH METHYL THIOURACIL AND OF CONTROL GROUPS AT BIRTH

Values given are means and standard deviations of the means. Number of animals in each group given in parenthesis

Dose Rate of Methyl Thiouracil (g/day)	Estimated Length of Gestation Period (days)	Weight of Ewes (lb)	Weight of Lambs (lb)	Body Length of Lambs (in.)	Length of Ossified Tibial Diaphysis of Lambs (in.)	Age of Lambs (length method) (days)	Osseous Maturity Score (units)	Age of Lambs (maturity method) (days)	Weight of Thyroid Glands of Ewes (g)	Weight of Thyroid Glands of Lambs (g)
0	146·6 ±2·2 (6)	120·0 ±2·5 (6)	7·63 ±0·67 (9)	27·12 ±0·81 (9)	3·73 ±0·12 (9)	147 ±4·0 (9)	39 ±0 (9)	143 ±0 (9)	3·55 ±0·03 (6)	0·93 ±0·12 (9)
0·5	156·8 ±1·1 (6)	129·0 ±3·6 (6)	6·69 ±0·41 (9)	25·53 ±0·58 (9)	3·27 ±0·13 (9)	132 ±2·7 (9)	22·8 ±2·5 (9)	122 ±3·2 (9)	33·7 ±6·5 (6)	69·8 ±14·3 (9)
1·5	169·5 ±3·5 (2)	124·5 ±0·5 (2)	6·72 ±0·04 (2)	25·35 ±0·15 (2)	3·25 ±0·01 (2)	131 ±0·2 (2)	13·0 ±1·0 (2)	109 ±1·2 (2)	34·0 ±8·1 (2)	56·5 ±16·9 (2)
4·5	161 (1)	112 (1)	4·38 ±0·30 (2)	23 ±1 (2)	2·42 ±0·02 (2)	113 ±0·6 (2)	8 ±0 (2)	103 ±0 (2)	11·8 (1)	18·15 ±8·15 (2)

woody texture compatible with the complete acinar collapse which was observed histologically. Van Gieson staining did not demonstrate any differences in collagen content.

Macroscopically the goitrous thyroid glands were deep red in colour and fleshy in texture and microscopically the epithelial cells were enormously hypertrophied; the acini had lost their normal shape and had become convoluted as a result of cellular crowding. Colloid which was abundant in the thyroid glands of the controls was present in small amounts only or completely absent in the glands of the treated groups. The other outstanding and constant features found at the autopsy were the enlarged fatty livers and kidneys.

The lambs in the treated groups showed more pronounced goitres than their dams. The goitrous thyroids of lambs differed from those of the ewes in that gross cystic changes had occurred; other histological changes were similar. The tongue and neck were usually somewhat oedematous in the lambs of the treated groups, and the jugular veins were engorged with blood. In addition, the trachea and larynx were distinctly compressed by the goitre and the pericardial sac usually contained a little fluid.

The wool covering of the lambs in the 0·5- and 1·5-g groups was coarse and hairy. In some cases the skin was only sparsely covered with hair, particularly in the dorsal half of the body, ears, face, and inguinal region. The two lambs of the surviving ewe from the 4·5-g group were hairless except for a few hairs on the muzzle, eyelids, horn buds, ventral aspect of the neck and underline to the umbilicus, plantar aspect of the tarsus and metatarsus, dorsal aspect of the carpus, and around the coronets. These findings are in accord with those of Ferguson *et al.* (1956) who described a delay in the maturity of secondary wool follicles in thyroidectomized lambs. The coarser wool observed in the lambs in the present study would indicate the presence of a higher proportion of primary follicles than normal.

(*b*) *Radiological Observations*

The length of the ossified tibial diaphysis was significantly smaller ($P < 0{\cdot}01$) in the treated groups.

There was a pronounced delay in the appearance of ossification centres in the treated lambs (Plates 1 and 2) and this is also represented in Table 1 under the heading "Osseous Maturity Score". Osseous maturity as assessed by counting the centres of ossification (Lascelles 1959) has a maximum score of 39 which is attained in the average normal Peppin-type Merino several days before birth. Thus it is not surprising to find that in the present data the lambs in the control group all have an osseous maturity score of 39. The difference between the scores of the controls and the treated groups is very large and is highly significant ($P < 0{\cdot}001$).

The epiphyseal and round bone centres of ossification in the hypothyroid lambs often first appeared as irregular fragmented areas of opacity on the radiograph, whereas in the normal lamb the centres appeared as distinct areas of uniform opacity.

TABLE 2

BIOCHEMICAL ESTIMATES MADE ON PLASMA SAMPLES TAKEN DURING PREGNANCY FROM EWES TREATED WITH METHYL THIOURACIL AND FROM CONTROL EWES

Values given are means and standard deviations of the means. Numbers of animals in each group given in parenthesis

Date of Collection of Blood	Dose Rate of Methyl Thiouracil (g/day)	PBI (mg/100 ml)	Cholesterol (mg/100 ml)	Phospholipid (mg/100 ml)	Total Esterified Fatty Acids (μ-equiv/ml)	Calcium (mg/100 ml)
27.v.58	0	11·2 ± 1·0 (6)	85 ± 7 (6)	88 ± 11 (6)	4·1 ± 0·5 (6)	11·2 ± 0·5 (6)
	0·5	10·2 ± 1·1 (6)	92 ± 14 (6)	91 ± 4 (6)	3·6 ± 0·4 (6)	10·0 ± 0·3 (6)
	1·5	9·8 ± 0·7 (6)	101 ± 6 (6)	79 ± 6 (6)	4·8 ± 0·6 (6)	10·2 ± 0·5 (6)
	4·5	11·0 ± 1·6 (6)	86 ± 6 (6)	78 ± 3 (6)	4·5 ± 0·4 (6)	10·9 ± 0·9 (6)
4.vii.58	0	8·0 ± 0·5 (6)	73 ± 6 (6)	66 ± 7 (6)	3·5 ± 0·3 (6)	12·9 ± 0·2 (6)
	0·5	5·1 ± 0·6 (5)	70 ± 6 (6)	63 ± 10 (5)	2·7 ± 0·3 (5)	12·7 ± 1·0 (5)
	1·5	6·6 ± 1·2 (5)	72 ± 8 (5)	82 ± 7 (4)	3·6 ± 0·1 (5)	11·4 ± 0·2 (5)
	4·5	6·5 ± 0·7 (5)	90 ± 6 (5)	72 ± 11 (5)	3·5 ± 0·5 (5)	11·5 ± 0·3 (5)
21.viii.58	0	10·8 ± 0·7 (6)	99 ± 3 (6)	80 ± 4 (6)	4·6 ± 0·1 (6)	12·9 ± 0·8 (6)
	0·5	8·9 ± 0·9 (6)	112 ± 6 (6)	82 ± 5 (6)	4·5 ± 0·1 (6)	10·6 ± 0·3 (6)
	1·5	6·4 ± 0·4 (4)	127 ± 12 (4)	93 ± 6 (4)	4·4 ± 0·2 (4)	10·0 ± 0·6 (4)
	4·5	4·9 (1)	140 (1)	123 (1)	5·7 (1)	8·4 (1)

The shafts of the long bones were unmodelled; the appearance of the normal central diaphyseal constriction and the development of the contours characteristic of normally maturing bone were retarded. In addition the medullary cavities were poorly hollowed out (Plate 3) and the general trabeculation of the bone in the appendicular skeleton was coarse.

Another outstanding and constant feature observed was the presence of transverse bands of increased density found towards the ends of the diaphysis (Plate 3). Such a phenomenom has been described in new-born babies and children (Caffey 1945); however, this is not a characteristic feature of cretinism in young children. Harris (1933) claimed that maternal illness during pregnancy could induce the formation of transverse bands in the foetal skeleton. This would suggest that the phenomenon observed in the present study was not a specific feature of hypothyroidism but was more likely to be associated with the setback of the ewe caused by the administration of the drug.

The ages of the hypothyroid lambs as determined by using the regression formula based on osseous maturity and tibial length measurements (Lascelles 1959) are set out in Table 1. There is a significantly greater reduction ($P < 0 \cdot 001$) in age (maturity) than in age (length).

(c) *Protein-bound Iodine and Thyroid Iodine*

Although the PBI levels of the ewes of the control group were all lower than those of their lambs, this difference was not significant, probably due to the small number of specimens. The mean concentrations of PBI for the foetuses is similar to that for new-born calves found by Lewis and Ralston (1953) who also found higher values for animals 0–48 hr old than for adult animals. There was no significant difference between the concentrations of PBI in the control and treated groups of either ewes or lambs. In the lambs, however, there was a highly significant difference ($P < 0 \cdot 001$) between the concentrations in the pooled control and lowest dose groups and the pooled values of the highest dose groups.

(d) *Plasma Lipid Concentrations*

The concentration of phospholipid in the plasma of the ewes of the control group at birth was significantly higher ($P < 0 \cdot 01$) than that of the foetuses. Although each ewe of the control group had higher concentrations of cholesterol and total esterified fatty acids than their lambs these differences just failed to be significant, probably due to the small number of specimens.

There was a pronounced and significant rise in the levels of the lipid constituents of the lamb caused by the methyl thiouracil treatment. Although the same tendency was apparent in the ewes, the differences were not significant. A significant increase in the maternal plasma cholesterol concentration of the treated groups was found at the last bleeding during pregnancy (Table 2). The failure to show a significant increase in the maternal plasma cholesterol concentration at birth is probably due to the loss of two samples from the control group.

TABLE 3

BIOCHEMICAL ESTIMATIONS MADE ON PLASMA AND THYROID SAMPLES TAKEN AT PARTURITION FROM EWES AND LAMBS TREATED WITH METHYL THIOURACIL AND OF CONTROL GROUPS

Values given are means and standard deviations of the means. Numbers of animals in each group given in parenthesis

Dose Rate of Methyl Thiouracil (g/day)	PBI Estimations (mg/100 ml)		Total Thyroid Iodine (mg)		Thyroid Iodine (% dry wt.)		Plasma Concentration of:							
							Phospholipids (mg/ml)		Cholesterol (mg/ml)		Total Esterified Fatty Acids (m-equiv/ml)		Calcium (mg/100 ml)	
	Ewe	Lamb	Ewe	Lamb	Ewe	Lamb	Ewe	Lamb	Ewe	Lamb	Ewe	Lamb	Ewe	Lamb
0	8·7 ±0·4 (4)	14·7 ±2·6 (5)	2·77 ±0·59 (6)	0·52 ±0·099 (7)	0·32 ±0·04 (7)	0·35 ±0·08 (7)	65·5 ±4·2 (4)	37·1 ±1·7 (5)	91·1 ±9·5 (4)	57·2 ±11·1 (5)	5·5 ±0·7 (4)	3·3 ±0·32 (4)	9·9 ±2·35 (4)	15·2 ±1·45 (5)
0·5	8·1 ±1·3 (6)	16·6 ±2·4 (8)	0·289 ±0·083 (6)	0·43 ±0·13 (7)	0·003 ±0·0004 (6)	0·007 ±0·0006 (7)	88·0 ±3·5 (6)	77·4 ±3·0 (8)	114 ±9·1 (6)	128 ±20 (7)	7·8 ±0·5 (6)	10·8 ±0·56 (8)	9·7 ±0·50 (6)	13·0 ±0·45 (8)
1·5	7·6 (1)	6·6 (1)	0·064 ±0·056 (2)	<0·01 (2)	0·001 ±0·001 (2)	<0·0001 (2)	80·5 ±8·5 (2)	102·5 ±18·5 (2)	100 ±8·0 (2)	156 ±9·0 (2)	6·3 ±1·75 (2)	10·3 ±0·85 (2)	6·4 ±0·65 (2)	4·4 ±0·65 (2)
4·5	4·1 (1)	6·7 ±0·1 (2)	0·060 (1)	<0·005 (2)	0·002 (1)	<0·0001 (2)	340 (1)	76·5 ±7·5 (2)	174 (1)	146 ±6·0 (2)	5·7 (1)	9·1 ±0·55 (2)	4·7 (1)	12·5 ±0·25 (2)

An increase in plasma cholesterol of ewes fed thiouracil has been reported by Barrick, Andrews, and Beeson (1950) and of other species treated with methyl thiouracil (Parodi and de Gregori 1948; Schettler 1950; Barr *et al.* 1955), although thiouracil did not affect the serum cholesterol concentration of rhesus monkeys (Aranow, Engle, and Sperry 1946).

There is a significant reduction ($P < 0 \cdot 001$) in the concentration of the thyroid iodine of both ewe and foetus in all treated groups. However, expressed as total thyroid iodine the difference between the control and lowest dose group is less in the ewes although still highly significant and there is no significant difference between the total thyroid iodine of the foetuses in the control and lowest dose group. The total thyroid iodine of the two highest dose groups is much less ($P < 0 \cdot 001$) than the control group for both ewe and foetus.

(*e*) *Plasma Calcium Concentration*

A decrease ($P < 0 \cdot 001$) in foetal plasma calcium concentration was found in the treated groups. A decrease ($P < 0 \cdot 01$) in maternal plasma calcium concentrations in the treated groups was shown at the last bleeding during pregnancy (Table 2) but the difference in the ewes at birth was not significant due to the loss of the two samples from the control group. The decreased plasma calcium concentration is in accord with the findings of Hall and Harvey (1952) but none of the sheep in the present work developed clinical hypocalcaemia. The biochemical results for ewe and foetus at parturition are summarized in Table 3.

IV. DISCUSSION

Osseous development can conveniently be divided into growth and maturity. These data demonstrate clearly that growth and maturity are separable entities as hypothyroidism has delayed the process of maturity or differentiation of the bone to a significantly greater degree than it has delayed bone growth.

From the point of view of field diagnosis of hypothyroidism in lambs the most satisfactory and specific factor would seem to be a change in thyroid weight and iodine concentration (with the derived total thyroid iodine giving an indication of the severity of the condition). Of equal importance would be the changes in skeletal maturity which, as far as is known, are specific.

PBI seems to be of little value as it tends to be correlated with total thyroid iodine rather than thyroid concentration and consequently would be affected only in more severe cases. This is a little difficult to reconcile with the undoubted hypothyroid changes seen in skeletal development and lipid metabolism as PBI has been assumed to be a measure of circulatory thyroid hormone. However, this interpretation may be affected by the fact that the sheep in this experiment were drenched only once a day, blood samples being collected at the time of drenching. As Ely, Olsen, and Reineke (1948) showed that methyl thiouracil is eliminated within 24 hr from calves amd goats, it is possible that a diurnal variation of PBI concentration may be minimizing the extent of the changes seen. Unfortunately this point was not examined. At least of corroborative value are the lipid estimations and, although these are much less specific, changes in lipid concentration are considered by some authors as an inevitable accompaniment of hypothyroidism in man (Mason, Hunt, and Hurxthal 1930; Boyd and Connell 1936, 1937; Gildes, Man, and Peters 1939), in the dog (Entenman, Chaikoff, and Reichert 1942; Schmidt and Hughes 1938; Thompson and Lond 1941) and in the rhesus monkey (Fleischmann, Schumaker, and Strauss 1943).

It is probably, *in part*, a reflection of the mobilization of body fat reserves caused by increased thyrotropic hormone secretion induced by the hypothyroidism (Asboe-Hansen 1958). In addition, disturbance of hepatic lipid metabolism occurs. Both a decrease in synthesis (Lipsky 1955) and a decrease in excretion (Rosenman, Friedman, and Byres 1951) of hepatic cholesterol have been reported.

V. Acknowledgments

The encouragement and advice of Emeritus Professor R. M. C. Gunn and Dr. P. J. Claringbold are greatly appreciated. Invaluable technical assistance was given by Mr. R. Layland and Mr. K. Bowlay. The work was assisted by a grant from the Wool Research Committee (to A.K.L.).

VI. References

Aranow, H., Engle, E. T., and Sperry, W. M. (1946).—*Endocrinology* **38**: 331.

Asboe-Hansen, G. (1958).—*Physiol. Rev.* **38**: 446.

Astwood, E. B. (1943).—*J. Pharmacol.* **78**: 79.

Barker, M. H., Lindberg, H. A., and Wald, M. H. (1941).—*J. Amer. Med. Ass.* **117**: 1591.

Barr, D. P., Russ, E. M., Elder, H. A., Kendall, F. E., and Abell, L. L. (1955).— *Circulation Res.* **3**: 199.

Barrick, E. R., Andrews, F. N., and Beeson, W. M. (1950).—*J. Anim. Sci.* **9**: 3.

Berson, S. A., and Yalow, R. S. (1955).—*J. Clin. Invest.* **34**: 186.

Boyd, E. M., and Connell, W. F. (1936).—*Qd. J. Med.* **5**: 455.

Boyd, E. M., and Connell, W. F. (1937).—*Qd. J. Med.* **6**: 467.

Brown-Grant, K., and Gibson, J. B. (1955).—*J. Physiol.* **127**: 328.

Caffey, J. (1945).—"Pediatric X-ray Diagnosis." (The Year Book Publishers Inc.: Chicago.)

Dun, R. B. (1955).—*Aust. Vet. J.* **31**: 153.

Ely, R. E., Olsen, K. J., Reineke, E. P. (1948).—*J. Anim. Sci.* **7**: 208.

Entenman, C., Chaikoff, I. L., and Reichert, F. L. (1942).—*Endocrinology* **30**: 794.

Ferguson, K. A., Schinckel, P., Carter, H., and Clarke, W. H. (1956).—*Aust. J. Biol. Sci.* **9**: 575.

Fleischmann, W., Schumaker, H. B., and Strauss, W. L. (1943).—*Endocrinology* **32**: 238.

Flux, D. S., Butler, G. W., Johnson, June M., Glending, A. L., and Peterson, G. B. (1956).—*N.Z.J. Sci. Tech.* A **38**: 88.

Franklin, M. C. (1931).—Rep. Dir. Ins. Anim. Path. No.2. p. 134.

Gildes, E. F., Man, E. B., and Peters, J. P. (1939).—*J. Clin. Invest.* **18**: 739.

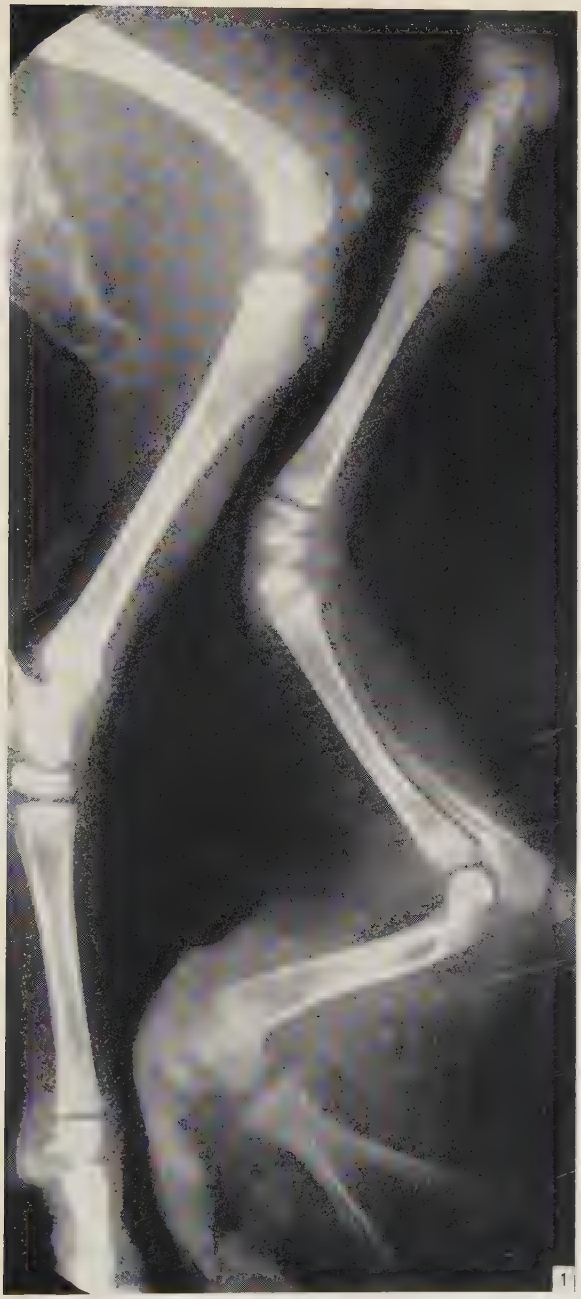
Gilruth, J. A. (1901).—Annu. Rep. Dep. Agric. N.Z. p. 214.

Grossman, A., and Grossman, G. F. (1955).—*J. Clin. Endocrinol.* **15**: 354.

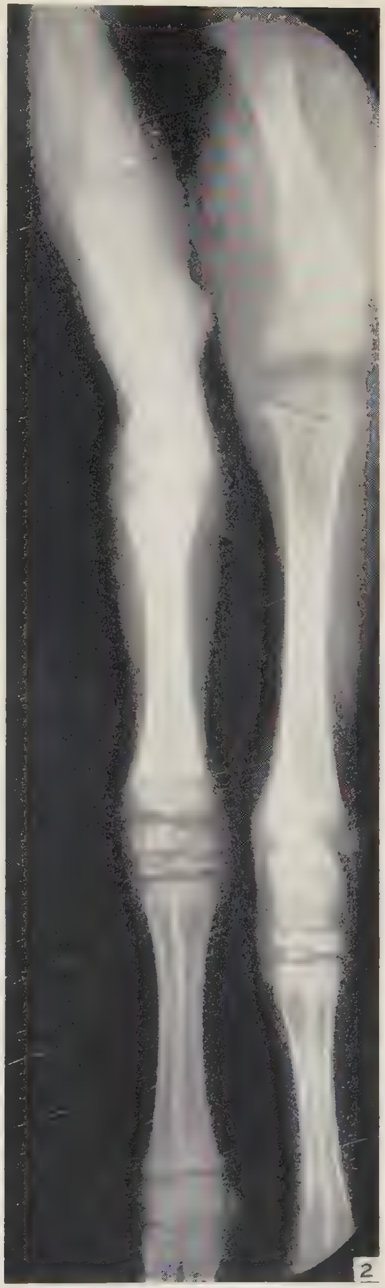
Hall, W., and Harvey, J. (1952).—*Qd. J. Agric. Sci.* **9**: 307.

Harper, T. A. (1959).—*Nature* **183**: 232.

HYPOTHYROIDISM IN SHEEP

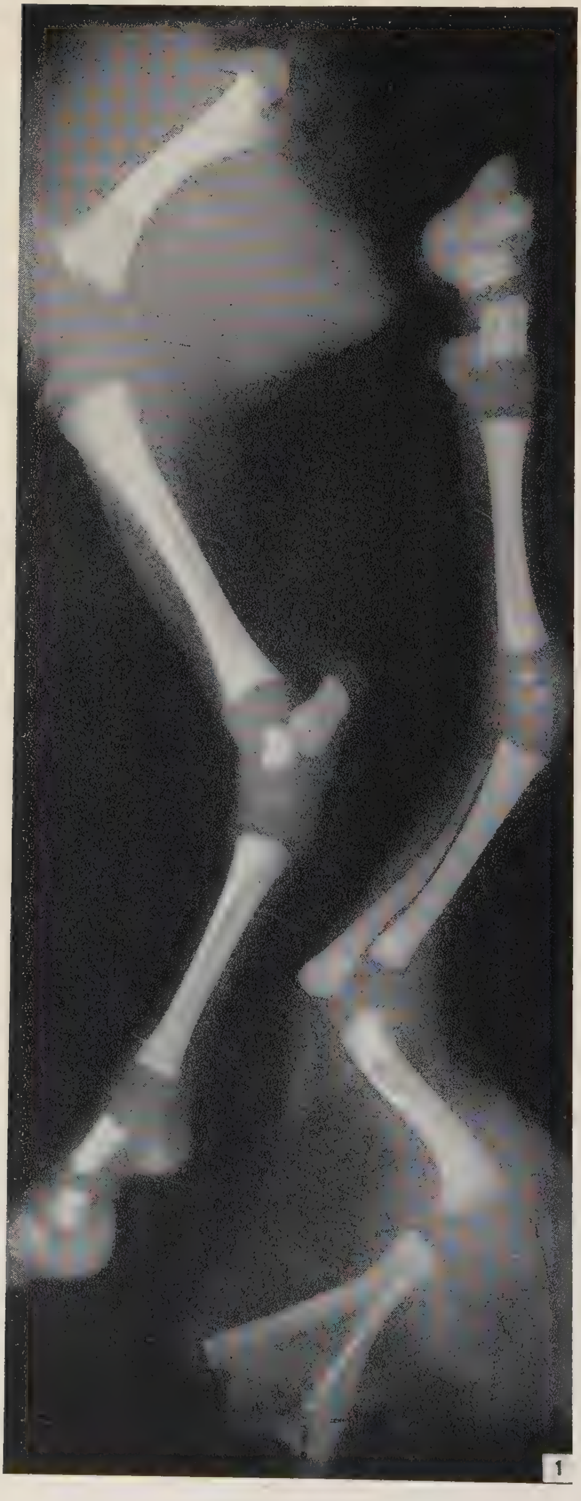


1

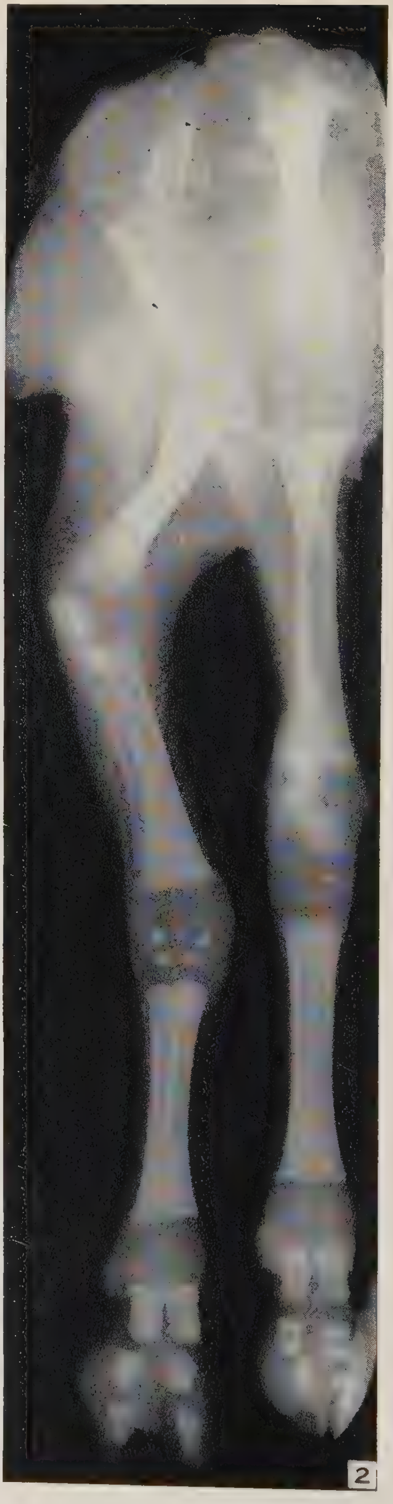


2

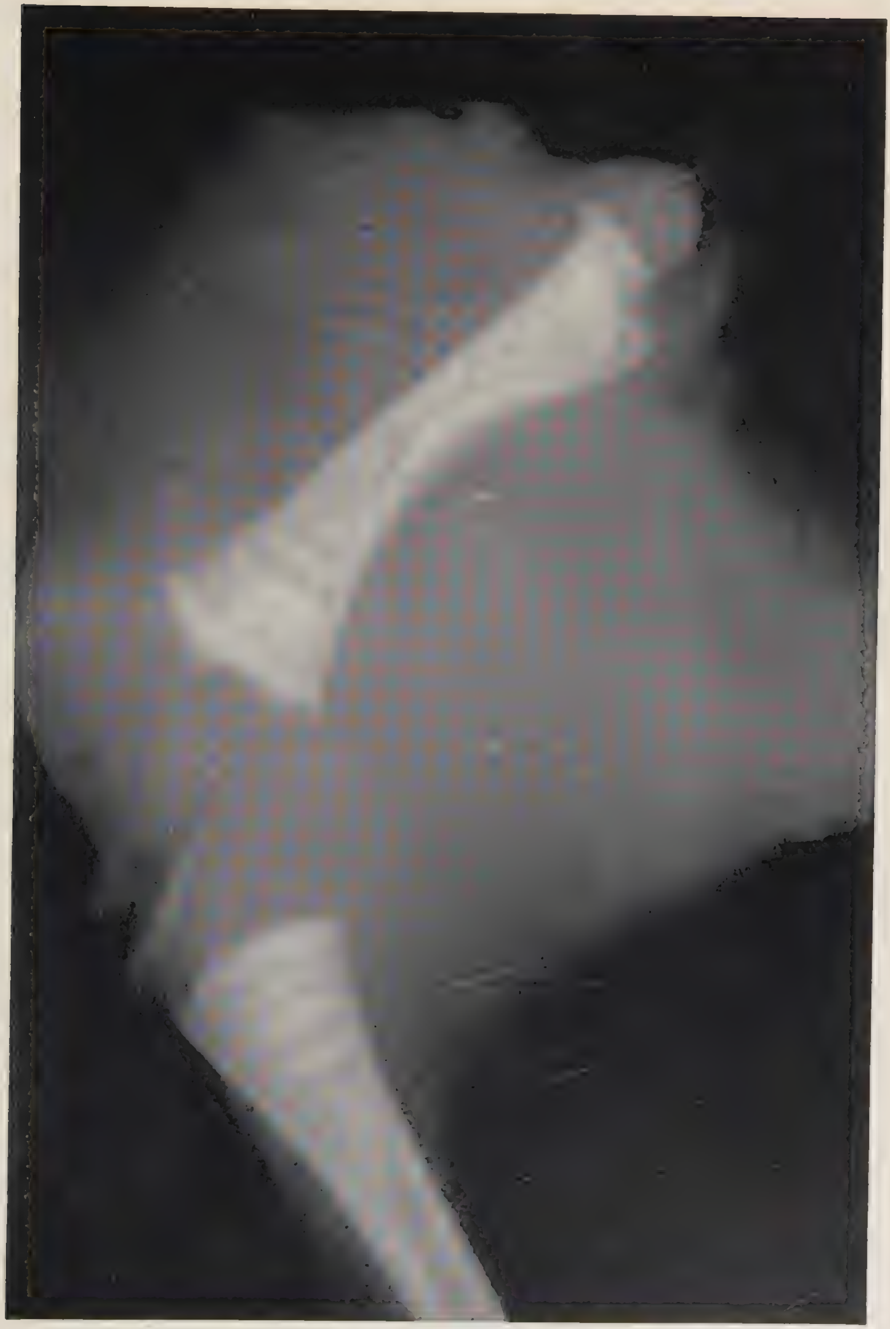
HYPOTHYROIDISM IN SHEEP



1



2



Harris, H. A. (1933).—"Bone Growth in Health and Disease." (Oxford Univ. Press: London.)

Hopkirk, C. S. M., Dayus, C. V., Simpson, B., and Grimmett, R. E. R. (1930).—*N. Z. J. Agric.* **58**: 226.

Lascelles, A. K. (1959).—*Aust. J. Zool.* **7**: 79.

Lewis, R. C., and Ralston, N. P. (1953).—*J. Dairy Sci.* **36**: 33.

Lipsky, S. R., Bondy, P. K., Man, E. B., and McGuire, J. S. Jr. (1955).—*J. Clin. Invest.* **34**: 950.

Mason, R. L., Hunt, H. M., and Hurxthal, I. (1930).—*New Engl. J. Med.* **203**: 1273.

Parodi, L., and Gregori, M. de (1948).—*Arch. Ital. Chir.* **70**: 199. (*Chem. Abstr.* **44**: 548 (1950).)

Pijoan, M., and Walters, C. W. (1937).—*J. Lab. Clin. Med.* **22**: 968.

Rawson, R. W., Tannheimer, J. F., and Peacock, W. (1944).—*Endocrinology* **34**: 245.

Rosenman, R. H., Friedman, M., and Byers, S. O. (1951).—*Science* **114**: 210.

Schettler, G. (1950).—*Z. Ges. Exp. Med.* **115**: 251.

Schmidt, L. H., and Hughes, H. B. (1938).—*Endocrinology* **22**: 474.

Setchell, B. P. (1959).—*Aust. J. Sci.* **21**(7): 221.

Simonsen, D. G., Wertman, M., Westover, L. M., and Mehl, J. W. (1946).—*J. Biol. Chem.* **166**: 747.

Sinclair, D. P., and Andrews, E. D. (1954).—*N.Z. Vet. J.* **2**: 72.

Stanley, M. M., and Astwood, E. B. (1948).—*Endocrinology* **42**: 107.

Stern, I., and Shapiro, B. (1953).—*J. Clin. Path.* **6**: 158.

Thompson, K. W., and Lond, C. N. H. (1941).—*Endocrinology* **28**: 715.

Todd, T. W., Wharton, R. E., and Todd, A. W. (1938).—*Amer. J. Anat.* **63**: 37.

Vanderlaan, J. E., and Vanderlaan, W. P. (1947).—*Endocrinology* **40**: 403.

Wolf, J., Chaikoff, I. L., Taurog, A., and Rubin, L. (1946).—*Endocrinology* **39**: 140.

Wright, E., and Sinclair, D. P. (1958).—*N.Z. J. Agric. Res.* **1**: 477.

Explanation of Plates 1–3

Plate 1

Radiographs, dorsal (Fig. 1) and lateral (Fig. 2) views, of the disarticulated fore and hind limb of the normal lamb demonstrating the extent of ossification at birth.

Plate 2

Radiographs, dorsal (Fig. 1) and lateral (Fig. 2) views, of disarticulated fore and hind limbs demonstrating grossly retarded ossification in a hypothyroid lamb at birth.

Plate 3

A lateral radiograph of the femero-tibial joint of a hypothyroid lamb showing transverse banding. In addition the narrowed medullary cavity and the unmodelled femoral diaphysis are obvious. No secondary centres of ossification have appeared in this region.

SOME EFFECTS OF FLUORIDE ON CALCIUM METABOLISM IN THE BONES OF YOUNG RATS

By J. R. Dunstone* and E. Payne†

[*Manuscript received April 27, 1959*]

Summary

Radioautography has indicated that in rats which were fed fluoride in the food at a rate of 400 p.p.m. (as sodium fluoride), and then injected with 17·6 μc ^{45}Ca, a process occurred which tended to cause a wider distribution of ^{45}Ca activity throughout the epiphyseal and trabecular regions of the femora and humeri. It also appeared that the labelled calcium was retained in these regions to a greater extent than in the control animals.

Radiochemical analyses showed that there was a tendency for the bones of fluoride-treated animals to have a greater overall ^{45}Ca activity, with significant increases in the lumbar and cervical vertebrae.

Few significant differences between the calcium content of the bones of fluoride-treated and control animals have been demonstrated, but there was some evidence to indicate that calcium is deposited and resorbed at a slower rate in fluoride-treated animals.

There appeared to be a tendency for the bones of fluoride-treated animals to have an ash content higher than that of the corresponding bones of the control animals, especially in the group of animals killed 10 days after the ^{45}Ca injection. There was also some evidence to indicate that ash-forming materials are deposited and resorbed at a slower rate in fluoride-treated animals.

Fluoride feeding has been shown to cause large increases in the bone fluoride level, with larger increases in the bones of animals exposed to fluoride feeding for the longer periods of time.

X-ray studies have shown no marked abnormalities in bone formation in fluoride-treated animals.

The humerus was the bone least affected by fluoride in all the experimental work.

I. Introduction

In Queensland, the high fluoride level of many waters is responsible for the occurrence of chronic endemic fluorosis in Merino sheep. An extensive study of this condition has been made by Harvey (1952, 1953*a*, 1953*b*), but the mechanisms by which fluoride exerts its deleterious effects are still relatively obscure.

Comar, Lotz, and Boyd (1952) and Comar *et al.* (1953) have studied the deposition and removal of ^{45}Ca in normal and fluoride-treated pigs using radioautographic techniques. They have reported that fluoride fed in the diet at concentrations of 200 and 1000 p.p.m. (as fluorine) caused a decrease in bone and body growth, and that a process occurs which tends to remove the ^{45}Ca originally deposited in the epiphyseal regions. They therefore suggested that fluoride caused

---

* Department of Biochemistry, University of Queensland.

† Department of Biochemistry, University of Queensland; present address: Animal Research Institute, Yeerongpilly, Qld.

increased bone resorption in these regions, the ^{45}Ca liberated in this process being utilized locally in the trabecular bone.

The bones of rats fed sodium fluoride at a rate of 4 mg per day for 10 weeks have been shown by Wadwhani (1954) to contain less nitrogen, more calcium, phosphate, carbonate, and sodium than those of control animals. The greatest differences between control and experimental animals were observed in the scapulae and the epiphyses. This author has suggested that fluoride affects the formation of collagen. No similar significant differences were observed when the experimental animals were fed sodium fluoride at a rate of 2 mg per day.

Kono (1954) has demonstrated a more active resorption in trabecular bone with rats fed fluoride at greater than 50 p.p.m. and a diminished resorption if the fluoride is fed at less than 10 p.p.m.

It has been reported by Arizono and Ichikawa (1955) as a result of histochemical studies that in rat fluorosis there is an increase in the calcification of dental pulp, bone shafts, trabeculae, and epiphyseal plates, the extent of the change being proportional to the dose and the period of administration of the fluoride.

Bélanger *et al.* (1958) have shown that in fluoride-treated pigs (1000 p.p.m. NaF), a rachitic condition similar to that caused by vitamin D deficiency occurs. Accompanying this condition they found decreased growth, imperfect mineralization, increased cartilage at the heads of the long bones, overproduction of osteoid, and increased acid-resistant ash which was only partly calcium fluoride.

The present study was commenced in order to investigate the effects of fluoride intake on the bones of young rats. X-ray, radioautographic, radiochemical, and purely analytical techniques were used in the hope that the results obtained would help in the understanding of the mechanism of fluoride toxicity and in the development of suitable methods for the further study of endemic fluorosis in Merino sheep.

II. Material and Methods

Thirty female rats, 7 weeks old and born on the same day and weighing 70–90 g, were paired by weight and placed on a basal diet of sucrose (70 per cent.), casein (18 per cent.), fat (5 per cent.) vitamin powder (Cuthbertson 1957) (1 per cent.) salt mixture (5 per cent.) and "Vetemul" (Nicholas Pty. Ltd.) (1 per cent.). This diet contained 0·9 per cent. calcium and 0·6 per cent. phosphorus. The composition of the vitamin powder and salt mixture is detailed in Table 1.

One member of each pair was fed sodium fluoride (400 p.p.m.) mixed with the diet; thereafter, until killing, the animals were pair-fed so as to ensure equal intakes, except, of course, for fluoride. On the 30th and 31st days of feeding the animals (wt. 100–130 g) were each injected intraperitoneally with 2 ml of a solution of ^{45}CaCl$_2$ in isotonic CaCl$_2$ at pH 7·4, containing 4·4 μc ^{45}Ca (3·7 mg calcium) per ml. Groups of animals consisting of five randomly selected pairs were killed 18 hr (group A), 10 days (group B), and 30 days (group C) after the final injection. Immediately after death each animal was X-rayed from the dorsal and lateral positions. "Kodirex" no-screen X-ray film was used and processed by the methods recommended by the manufacturer.

The bulk of the adhering flesh was removed from all bones, care being taken to avoid damage. The bones were then autoclaved at a pressure of 20 lb/sq. in. for 10 min. After this treatment the remaining flesh was easily removed without damaging the bone.

The bones for radioautography were first dried at 80°C and then placed in activated acrylic monomer (Imperial Chemical Industries Ltd.) to which had been added a small quantity of benzoyl peroxide. The specimens were then placed in a vacuum desiccator which was then evacuated. This treatment enabled the embedding material to permeate the bone pores and marrow cavities. After about 30 min, the specimens were removed and cured at 60°C for 48 hr, after which time the embedding material had set hard. Approximately mid-sagittal ground hemisections were prepared by sectioning on a lathe and polishing flat. These sections were then pressed on to strips of "Kodirex" no-screen X-ray film attached to glass slides, wrapped in black paper, and set aside at 4°C for 24 hr. The radioautographs were then developed using the standard techniques recommended by the manufacturer for the particular film used.

TABLE 1

COMPOSITION OF THE VITAMIN POWDER AND SALT MIXTURE MOIETY OF THE BASAL DIET

Vitamin Powder	Content (mg)	Salt Mixture	Content (g)
Thiamine hydrochloride	100	$MgSO_4.7H_2O$	32·9
Riboflavin	100	NaCl	69
Pyridoxine	40	KCl	112
Calcium pantothenate	240	KH_2PO_4	212
Nicotinic acid	200	$MgCO_3$	25
Vitamin B_{12}	0·6	Ferric citrate	31
Choline chloride	20,000	$CaCO_3$	542·5
α-Tocopherol	1,000	KI	0·08
		$CuSO_4.5H_2O$	1·4
Made up to 200 g with sucrose		$MnSO_4.4H_2O$	0·4
		$CoSO_4.7H_2O$	0·03
		$NaH_2PO_4.2H_2O$	300

Bones required for chemical and radiochemical analysis were dried at 80°C, ground finely, rendered fat-free by Soxhlet extraction with petroleum ether (B.P. 60–80°C), and again dried at 80°C. These materials were then dry-ashed at 700–800°C, dissolved in 2N HCl, and diluted to 10 ml with distilled water.

Samples were prepared for radioactive counting by transferring 0·3-ml portions of these solutions to 24-mm planchets, neutralizing the excess acid with 15N NH_4OH, adding one drop of 10 per cent. (w/v) kaolin in alcohol to assist spreading, and drying under an infra-red lamp. The radioactive counting was carried out using an end-window Geiger–Müller tube in association with a standard recording and scaling unit. All samples were prepared in triplicate and, provided a sufficient number of counts were recorded (approx. 5000 for each sample) and corrections were made for self-absorption, the results were reproducible to within 4 per cent. of each other.

Calcium was determined in these solutions by taking suitable portions and titrating with ethylenediaminetetra-acetic acid, using murexide as indicator (Dunstone 1957).

Fluoride was determined in these solutions by the method described in a report of a Sub-Committee of the Analytical Methods Committee (Society of Public Analysts (Great Britain) 1944) as modified by Harvey (1952).

III. Results

The mean sodium fluoride intake for all experimental animals was $3 \cdot 7 \pm 0 \cdot 1$ mg per animal per day.

After the injection of ^{45}Ca, the animals showed no increases in body weight for about 2 weeks. Some animals actually lost weight immediately after the injection. This may have been caused by the relatively large amount of calcium that had to be injected because of the low specific activity of the ^{45}Ca available. A second reason may have been the large amount of ^{45}Ca administered ($17 \cdot 6$ μc). However, at all times during the experiment, the physical condition of control and experimental animal in each pair did not differ significantly.

Radioautographs have been made of one femur and one humerus from each animal; however, in the interest of economy of space, only selected radioautographs of the femora and humeri are presented. Similar radioautographs were obtained for corresponding animals of each group.

Figures 1 and 2 (Plate 1) represent typical radioautographs of the femora from a pair of animals from group A, while Figures 3 and 4 (Plate 1) and Figures 5 and 6 (Plate 1) represent the radioautographs of the femora from pairs of animals from groups B and C respectively.

Plate 1, Figure 1, the radioautograph of the femur of a control animal, shows a distinct line of deposition of the ^{45}Ca in the epiphyseal region. Plate 1, Figure 2, the corresponding radioautograph for a fluoride-treated animal is similar to Plate 1, Figure 1, but in this case the deposition of ^{45}Ca appears to be more diffuse and there seems to be greater deposition in the trabecular bone. In both treated and untreated animals there is considerable ^{45}Ca activity in the "funnel" region of the diaphysis and in the periosteal bone.

Figures 3 and 4 (Plate 1) respectively show the radioautographs of the femora of control and fluoride-treated animals killed 10 days after ^{45}Ca injection. Figure 4 differs from Figure 3 in that there appears to be a wider distribution of ^{45}Ca activity throughout the epiphyseal and trabecular regions with greater overall activity. The areas of activity in the funnel region of the diaphysis and the periosteal bone are still present in both treated and untreated animals.

TABLE 2

EFFECT OF FLUORIDE FEEDING ON ^{45}Ca CONTENT OF BONES

Mean values ± S.E. for the specific activity of the ^{45}Ca are given for the results, which are expressed as a percentage of the administered dose per milligram of calcium

Bone	Group	No. of Animals	Control (% × 10^3)	Fluoride-treated (% × 10^3)
Femur	A	4	54 ± 6	55 ± 6
	B	4	53 ± 2	51 ± 3
	C	4	43 ± 2	47 ± 2
Humerus	A	4	58 ± 4	60 ± 4
	B	4	52 ± 6	59 ± 1
	C	5	41 ± 1	41 ± 2
Lumbar vertebra	A	4	63 ± 3	63 ± 5
	B	5	68 ± 4	73 ± 2
	C	5	46 ± 3	55 ± 1
Cervical vertebra	A	4	51 ± 4	63 ± 4
	B	5	68 ± 4	76 ± 5
	C	5	57 ± 3	60 ± 4
Mandible	C	5	58 ± 3	62 ± 3

Analysis of Variance

Bone	Source of Variation	Degrees of Freedom	Sum of Squares (× 10^6)	Mean Square (× 10^6)	Variance Ratio
Femur	Mean response	1	48	48	1·37
	Between groups	2	152	76	2·18
	Within groups	9	312	35	—
Humerus	Mean response	1	105	105	1·01
	Between groups	2	187	93·5	0·90
	Within groups	10	1035	103·5	—
Lumbar vertebra	Mean response	1	293	293	8·9*
	Between groups	2	487	244	7·4**
	Within groups	11	360	33	—
Cervical vertebra	Mean response	1	729	729	6·18*
	Between groups	2	871	435	3·69
	Within groups	11	1299	118	—

Test of Significance of Difference between Groups

Bone		Mean Difference Required for Significance at: 5% Level	1% Level	Groups	Mean Difference between Groups
Lumbar vertebra	Means of 4 and 5	0·0085	0·0121	A and B	0·0056
	Means of 5 and 5	0·0080	0·0113	B and C	0·0038
				A and C	0·0094

* $P < 0·05$. ** $P < 0·01$.

Little ^{45}Ca activity is apparent in the epiphyseal regions of the femora from animals of the third group (Plate 1, Fig. 5 (control) and Plate 1, Fig. 6 (fluoride-treated)). What little activity there is appears to be greater in the case of fluoride-treated animals especially in the region of trabecular bone. The characteristic areas of deposition in the funnel region of the diaphysis and in the periosteal bone are still present, and practically identical with those of the radioautographs from the animals of the first two groups.

Similar patterns were observed with the humeri, but differences between control and fluoride-treated animals were not so apparent (Plate 2, Figs. 7–12). Here, the radioautographs of the humeri show considerable ^{45}Ca deposition in the posterior periosteal and in the anterior endosteal regions of the bone shafts, whereas the radioautographs of the femora show only periosteal deposition in the bone shafts. Similar observations have been made by Tomlin, Henry, and Kon (1953).

The results of the radioactivity measurements on the femora, humeri, vertebrae, and mandibles (group C only) are given in Table 2. An appropriate statistical treatment of the experimental data has been carried out and included in the table. The specific activity of the ^{45}Ca is expressed as a percentage of the original dose administered per milligram of calcium present in the bone.

No significant differences between the ^{45}Ca activities of the femora and humeri of fluoride-treated and control animals were observed, but the activities of the lumbar and cervical vertebrae of fluoride-treated animals were significantly higher than those of the control animals (lumbar vertebrae, mean response $P < 0{\cdot}05$; cervical vertebrae, mean response $P < 0{\cdot}05$). A significant difference between groups was also found for the lumbar vertebrae ($P < 0{\cdot}01$), the differences of activity between fluoride-treated and control animals being greatest in group C and least in group A; however, only the difference between groups A and C was statistically significant at the 5 per cent. level. An interesting observation with respect to the metabolism of ^{45}Ca is that while the femur and humerus retain an approximately constant specific activity for a period of 10 days followed by a decrease, the vertebrae show a considerable increase in specific activity over the initial 10 days followed by a decrease similar to that observed for the femur and humerus.

Table 3 summarizes the results of the calcium analyses of the various bones. No significant differences between the values for control and fluoride-treated animals could be demonstrated, except in the case of the cervical vertebrae where the fluoride-treated animals showed a significantly lower calcium content (mean response $P < 0{\cdot}05$). Significant differences between groups were obtained for the femora ($P < 0{\cdot}05$), and the cervical vertebrae ($P < 0{\cdot}05$). Mean differences required for significance between any two of the groups show that the differential effect between groups A and B and groups A and C of the femora are significant at the 1 per cent. and 5 per cent. levels respectively, while the differential effect between groups A and B and groups B and C of the cervical vertebrae are significant at the 5 per cent. level. In the 9-day period between the killing of groups A and B, there appears to be a decrease in the calcium content of bones of all animals, followed by an increase in the following 20 days. Both the initial decreases and subsequent increases are

Table 3

EFFECT OF FLUORIDE FEEDING ON THE CALCIUM CONTENT OF BONES

Mean values ± S.E. are given for the percentage calcium in the water- and fat-free bones

Bone	Group	No. of Animals	Control (%)	Fluoride-treated (%)
Femur	A	4	24·0 ± 0·4	23·2 ± 0·4
	B	4	23·1 ± 0·4	23·9 ± 0·2
	C	4	24·3 ± 0·1	24·6 ± 0·4
Humerus	A	5	24·3 ± 0·1	24·2 ± 0·1
	B	4	24·4 ± 0·8	24·7 ± 0·4
	C	5	25·3 ± 0·5	25·4 ± 0·5
Lumbar vertebra	A	4	22·1 ± 0·4	22·9 ± 0·6
	B	5	21·0 ± 0·3	21·6 ± 0·4
	C	5	23·0 ± 0·4	22·8 ± 0·2
Cervical vertebra	A	5	23·7 ± 0·3	23·1 ± 0·3
	B	5	22·3 ± 0·3	22·5 ± 0·2
	C	5	23·4 ± 0·3	22·7 ± 0·1
Mandible	C	5	26·6 ± 0·3	26·1 ± 0·3

Analysis of Variance

Bone	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Femur	Mean response	1	0·163	0·163	0·31
	Between groups	2	4·77	2·39	4·6*
	Within groups	9	4·69	0·52	—
Humerus	Mean response	1	0·121	0·121	0·13
	Between groups	2	0·462	0·231	0·26
	Within groups	11	9·887	0·90	—
Lumbar vertebra	Mean response	1	2·49	2·49	3·61
	Between groups	2	5·20	2·60	3·77
	Within groups	11	7·56	0·69	—
Cervical vertebra	Mean response	1	1·73	1·73	6·65*
	Between groups	2	3·93	1·96	7·54**
	Within groups	12	3·09	0·26	—

Test of Significance of Differences between Groups

Bone		Mean Difference Required for Significance at:		Groups	Mean Difference between Groups
		5% Level	1% Level		
Cervical vertebra	Means of 5 and 5	0·70	0·99	A and B	0·78
				B and C	0·84
				A and C	0·06
Femur	Means of 4 and 4	1·05	1·48	A and B	1·48
				B and C	0·42
				A and C	1·06

*$P < 0\cdot05$. **$P < 0\cdot01$.

somewhat smaller in the fluoride-treated animals, indicating a slower resorption and uptake of calcium in these animals. The humerus is the only bone that does not follow this pattern.

The percentage ash of the water- and fat-free bones of fluoride-treated and control animals is shown in Table 4. Only the lumbar vertebrae show a significant difference ($P < 0 \cdot 01$) between the percentage ash of fluoride-treated and control animals, the treated animals having the higher values. Significance between groups was obtained for lumbar vertebrae ($P<0 \cdot 01$), and the cervical vertebrae ($P<0 \cdot 05$).

In the case of the lumbar vertebrae, the differences between the percentage ash of control and fluoride-treated animals was greatest in group A and least in group C, the difference between groups A and C being significant at the 5 per cent. level. With the cervical vertebrae, the greatest differences in percentage ash between fluoride-treated and control animals occurred in group B, the difference between groups B and C being significant at the 5 per cent. level. In the 9-day period between the killing of groups A and B, there appears to be a decrease in the ash content of bones of all animals, followed by an increase in the subsequent 20 days. Both the initial decreases and subsequent increases are somewhat smaller in the fluoride-treated animals, indicating a slower release and uptake of ash-forming materials in the fluoride-treated animals. Again the only bone that does not follow this pattern is the humerus.

The fluoride content of the water- and fat-free bones is shown in Table 5. The bones from all animals were not examined, but the results indicate that the bones of fluoride-fed animals contain 15–20 times more fluoride than those of the control animals. The difference in fluoride content between the bones of fluoride-treated and control animals is greatest in group C, and least in group A. The observed differences were of such magnitude that statistical analyses were not carried out.

No gross abnormalities in bone structure, such as the formation of exostoses, bone thickening, etc. could be detected in the X-rays of fluoride-treated animals when compared with those of the control animals.

IV. Discussion

The radioautographs obtained from the femora of the animals of group A (killed 18 hr after final injection) were similar to those obtained by Comar *et al.* (1953) for pigs killed 5 hr after ^{45}Ca injection. This would then verify the fact observed by these authors that in both fluoride-treated and untreated animals there is initial deposition of ^{45}Ca in the epiphyseal region with the fluoride-treated animals showing more diffuse deposition in this and in the trabecular regions.

In the radioautographs of the femora and humeri from the second group of animals, there appeared to be a wider distribution of ^{45}Ca in the area immediately below the epiphysis in the fluoride-fed animals. It would also appear that the labelled calcium in the bones of fluoride-treated animals is retained in these regions to a greater extent than in the control animals.

The radioautographs obtained from control and fluoride-treated animals of the third group show that the line of initial epiphyseal deposition present in the

TABLE 4

EFFECT OF FLUORIDE FEEDING ON THE ASH CONTENT OF BONES

Mean values ± S.E. are given for the percentage ash of the water- and fat-free bones

Bone	Group	No. of Animals	Control (%)	Fluoride-treated (%)
Femur	A	4	64·4 ± 0·5	63·1 ± 0·7
	B	4	60·0 ± 0·3	61·9 ± 0·2
	C	4	62·1 ± 0·3	62·9 ± 0·3
Humerus	A	5	57·3 ± 0·5	56·5 ± 0·7
	B	4	63·4 ± 0·8	64·3 ± 0·1
	C	5	63·2 ± 0·4	63·5 ± 0·4
Lumbar vertebra	A	4	57·5 ± 0·3	59·6 ± 0·2
	B	5	54·7 ± 0·7	56·2 ± 0·4
	C	5	59·9 ± 0·4	60·5 ± 0·4
Cervical vertebra	A	5	60·4 ± 0·5	60·7 ± 0·4
	B	5	56·5 ± 0·6	58·2 ± 0·5
	C	5	61·1 ± 1·0	60·8 ± 0·4
Mandible	C	5	68·9 ± 0·8	69·5 ± 0·7

Analysis of Variance

Bone	Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	Variance Ratio
Femur	Mean response	1	2·43	2·43	0·74
	Between groups	2	23·22	11·61	3·56
	Within groups	9	29·39	3·26	—
Humerus	Mean response	1	0·4	0·4	0·072
	Between groups	2	4·59	2·30	0·41
	Within groups	11	61·26	5·57	—
Lumbar vertebra	Mean response	1	23·92	23·92	32·8**
	Between groups	2	28·86	14·43	19·8**
	Within groups	11	8·07	0·73	—
Cervical vertebra	Mean response	1	0·55	0·55	0·34
	Between groups	2	16·6	8·3	5·2*
	Within groups	12	19·32	1·60	—

Test of Significance of Difference between Groups

Bone		Mean Difference Required for Significance at:		Groups	Mean Difference between Groups
		5% Level	1% Level		
Lumbar vertebrae	Means of 4 and 5	1·26	1·69	A and B	0·60
	Means of 5 and 5	1·19	1·67	B and C	0·87
				A and C	1·47
Cervical vertebrae	Means of 5 and 5	1·75	2·26	A and B	1·40
				B and C	2·06
				A and C	0·66

*$P < 0\cdot05$. **$P < 0\cdot01$.

radioautographs of the bones from animals of the first two groups is absent in both control and fluoride-treated animals. This indicates a removal of the ^{45}Ca from these regions. Although only small differences between the radioautographs of the bones of control and fluoride-treated animals were observed, it appears that in the case of the treated animals more ^{45}Ca activity was present in the trabecular regions. Thus it is suggested that resorption of ^{45}Ca originally deposited in the epiphyseal region occurs in both control and fluoride-treated animals with a greater utilization of the removed calcium in the trabecular bone of the fluoride-treated animals.

This retention of ^{45}Ca activity, as visualized by the radioautographs, is supported to some extent by the radiochemical analyses which indicate that there is a tendency towards greater overall ^{45}Ca activity in the bones of fluoride-treated animals. The most pronounced increases in ^{45}Ca activity were observed in the vertebrae. This is not surprising, as it is a well-known fact that these bones are often seriously affected in fluorotic conditions (Harvey 1952). Significant differences between groups have only been demonstrated for the lumbar vertebrae and in this case the increase in ^{45}Ca activity for fluoride-treated animals is greatest in group C and least in group A (no difference between control and fluoride-treated animals in this group). It is therefore suggested that with these bones, and possibly with others, the rate of initial deposition of^{45}Ca is almost the same in both control and fluoride-treated animals, but the ^{45}Ca activity is retained to a greater extent in the fluoride-treated animals. Increased ^{45}Ca retention is contrary to the observations of Comar, Lotz, and Boyd (1952) and Comar *et al.* (1953) and it is difficult to find an explanation of how it might occur. One of a number of possibilities is that the retention might be the result of the formation in the matrix of calcium fluoride or an organic salt of calcium which is less easily removed than the usual hydroxylapatite crystal. Bélanger *et al.* (1958) have reported the occurrence of such compounds in the matrix between the cartilage cells in the bones of fluoride-treated pigs, the bones showing an increased acid-resistant ash. The tendency towards increased ash in our experiments also lends some support to this explanation.

Fluoride appeared to exert no significant overall effect on the calcium content of bones, except in the case of the cervical vertebrae where fluoride-treated animals showed a slightly decreased calcium content. These results do not agree with those obtained by Wadwhani (1954) who found an increased calcium content in the bones of rats fed 4 mg sodium fluoride per day for 10 weeks. His experiments would correspond to the group C experiments of this communication. The diet fed by Wadwhani contained 0·27 per cent. phosphorus and 0·38 per cent. calcium, whereas the diet used in these experiments contained 0·6 per cent. phosphorus and 0·9 per cent. calcium, and it is possible that the high level of dietary calcium may have influenced the effect of the fluoride. Several workers (Ranganathan 1944; Smith and Shaner 1944; Greenwood *et al.* 1946; Wadwhani 1954) report that high calcium levels in the diet decrease the effects of fluoride. On the other hand, Harvey (1952) and Suttie, Phillips, and Miller (1958) have found that high dietary calcium levels do not influence the effect of fluoride. In view of the fluoride content of the bones of the experimental rats of this study, it appears that the dietary calcium level has had little influence on the uptake of fluoride by the bones. The significant differential effect observed between groups for the calcium content of the femora and cervical vertebrae indicates that a slower resorption and subsequent uptake of calcium has occurred in the fluoride-treated animals. Although no significance could be demonstrated, the lumbar vertebrae showed a similar pattern, while the humerus showed little difference between fluoride-treated and control animals. This lends some support to the explanation that the greater retention of ^{45}Ca in bones of fluoride-treated animals is due to the formation of calcium fluoride or a calcium salt in the matrix, this compound being less easily resorbed than the usual hydroxylapatite crystal.

Table 5

EFFECT OF FLUORIDE FEEDING ON THE FLUORIDE CONTENT OF BONES

The results are expressed in p.p.m. of fluorine in water- and fat-free bone

Bone	Group	Control (p.p.m.)	Fluoride-treated (p.p.m.)
Femur	A	154	3090
	B	225	3780
	C	374	4810
Humerus	A	210	3460
	B	310	3950
	C	310	4210
Lumbar vertebra	A	155	3560
	B	317	3930
	C	299	4990
Cervical vertebra	A	148	3450
	B	309	3530
	C	291	4700
Mandible	C	385	4100

There appeared to be an overall tendency for the bones of fluoride-treated animals to have a greater ash content than those of the control animals, but this is by no means statistically significant. Smith and Lantz (1933) and Munoz (1936) have found that fluoride decreases the percentage ash of rat bones. Kick *et al.* (1935) have found that fluoride exerts no apparent effect on the ash content of pig bones, while McClure and Mitchell (1931) have reported that fluoride increases the ash content of rat bones. Peirce (1938) has shown slight increases in the ash content of the bones of fluoride-treated sheep but the values were still within the normal range. It appears that the increase in ash content of the bones of the fluoride-treated rats of these experiments follows a pattern similar to that observed by Peirce (1938) for sheep.

The significant differential effect observed between groups for the ash content of the lumbar and cervical vertebrae, with the effect for the femora being almost significant at the 5 per cent. level, indicates a slower resorption and subsequent uptake of ash-producing materials in fluoride-treated animals. This gives some support to the explanation previously given for calcium retention in fluoride-treated animals, if the ash-producing materials are compounds such as those suggested by Bélanger *et al.* (1958).

The decrease in the ash and calcium content of bones that occurred in the 9-day period between the killing of groups A and B was not expected and an explanation of its occurrence is not possible without further investigation.

No marked differences in the bones of control and fluoride-treated animals were observed by X-ray examination. Exostoses and other malformations of the bone, which occur in animals fed fluoride for a considerable period of time, did not appear in these experiments, presumably because of the shorter period of administration.

The results of the radiochemical and chemical analyses indicate that fluoride exerts a greater effect on the vertebrae, particularly the lumbar vertebrae, than on the long bones. No explanation of how these effects might occur is offered, but it is hoped that in the near future a more detailed study of these effects on the vertebrae will be commenced.

V. ACKNOWLEDGMENTS

The authors are indebted to Professor H. J. G. Hines, Dr. J. M. Harvey, Dr. C. C. Kratzing, and Dr. O. Budtz-Olsen for their advice and helpful criticism. Thanks are due to Dr. J. A. Sagar for assistance with the X-rays, Mr. K. P. Haydock for advice on statistical treatment, and Mr. E. Hollywood and Miss N. Bowyer for preparing the photographs. One of us (E.P.) wishes to thank C.S.I.R.O. the for the award of a Junior Post-Graduate Studentship.

VI. REFERENCES

ARIZONO, H., and ICHIKAWA, M. (1955).—*Med. J. Osaka Univ.* **6**: 205–16.

BÉLANGER, L. F., VISEK, W. J., LOTZ, W. E., and COMAR, C. L. (1958).—*Amer. J. Path.* **34**: 25–35.

COMAR, C. L., LOTZ, W. E., and BOYD, G. A. (1952).—*Amer. J. Anat.* **90**: 113–25.

COMAR, C. L., VISEK, W. J., LOTZ, W. E., and RUST, J. H. (1953).—*Amer. J. Anat.* **92**: 361–89.

CUTHBERTSON, W. F. J. (1957).—*Proc. Nutr. Soc.* **16**: 70–6.

DUNSTONE, J. R. (1957).—*Med. J. Aust.* **2**: 571–2.

GREENWOOD, D. A., BLAYNEY, J. R., SKINSNES, O. K., and HODGES, P. C. (1946).—*J. Dent. Res.* **25**: 311–26.

HARVEY, J. M. (1952).—*Qd. J. Agric. Sci.* **9**: 47–141.

HARVEY, J. M. (1953*a*).—*Qd. J. Agric. Sci.* **10**: 109–24.

HARVEY, J. M. (1953*b*).—*Qd. J. Agric. Sci.* **10**: 127–59.

KICK, C. H. ET AL. (1935).—Bull. Ohio Agric. Exp. Sta. No. 558.

KONO, K. (1954).—*Shikoku Acta Med.* **5**: 78–87.

MCCLURE, F. J., and MITCHELL, H. H. (1931).—*J. Biol. Chem.* **90**: 297–320.

MUNOZ, J. M. (1936).—*Rev. Soc. Argent. Biol.* **12**: 50–6. (*Biol. Abstr.* **11**: 8621 (1937).)

PEIRCE, A. W. (1938).—Bull. Coun. Sci. Industr. Res. Aust. No. 121.

RANGANATHAN, S. (1944).—*Indian J. Med. Res.* **32**: 233–6.

SMITH, M. C., and LANTZ, E. M. (1933).—*J. Biol. Chem.* **101**: 677–83.

SMITH, R. R., and SHANER, E. O. (1944).—*J. Amer. Dent. Ass.* **31**: 1483–6.

SOCIETY OF PUBLIC ANALYSTS (GREAT BRITAIN) (1944).—*Analyst* **69**: 243–6.

SUTTIE, J. W., PHILLIPS, P. H., and MILLER, R. F. (1958).—*J. Nutr.* **65**: 293–304.

TOMLIN, D. H., HENRY, K. M., and KON, S. K. (1953).—*Brit. J. Nutr.* **7**: 235–52.

WADWHANI, T. K. (1954).—*Proc. Indian Acad. Sci.* B **39**: 223–35.

Explanation of Plates 1 and 2

Plate 1

Figs. 1 and 2.—Radioautographs of the femora of control (Fig. 1) and fluoride-fed (Fig. 2) animals killed 18 hr after ^{45}Ca injection. Note the distinct line of deposition of ^{45}Ca in the epiphyseal region and the slightly greater ^{45}Ca activity in the trabecular region of the femur of the fluoride-treated animal.

Figs. 3 and 4.—Radioautographs of the femora of control (Fig. 3) and fluoride-fed (Fig. 4) animals killed 10 days after ^{45}Ca injection. Note the wider distribution of ^{45}Ca activity in the trabecular region of the femur of the fluoride-treated animal.

Figs. 5 and 6.—Radioautographs of the femora of control (Fig. 5) and fluoride-fed (Fig. 6) animals killed 30 days after ^{45}Ca injection. Note the absence of the line of original deposition of ^{45}Ca in both control and fluoride-treated animals. There appears to be slightly more ^{45}Ca activity in the trabecular bone of the fluoride-treated animal.

Plate 2

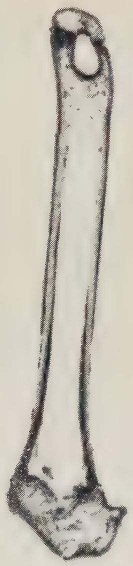
The radioautographs of the humeri of control and fluoride-fed animals do not show such marked differences as do those of the femora, but they exhibit the same essential features. Note the areas of endosteal and periosteal deposition of the ^{45}Ca

Figs. 7, 9, and 11.—Radioautographs of the humeri of control animals killed respectively 18 hr, 10 days, and 30 days after ^{45}Ca injection.

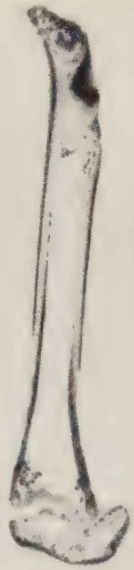
Figs. 8, 10, and 12.—Corresponding radioautographs of the humeri of fluoride-treated animals.

EFFECT OF FLUORIDE ON BONE METABOLISM

1



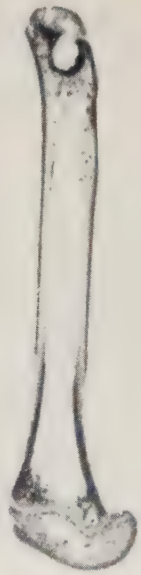
3



5

2

4



6

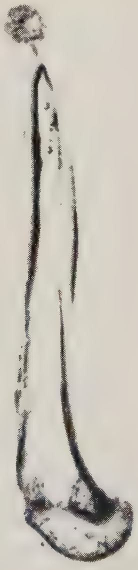
EFFECT OF FLUORIDE ON BONE METABOLISM

7

9

11

8



10

12

STUDIES OF CASEIN

V. THE ACTION OF RENNIN ON CASEIN

By R. G. Wake*

[*Manuscript received January* 30, 1959]

Summary

The liberation of non-protein nitrogen, soluble in 12 per cent. trichloroacetic acid, from various milk protein fractions by rennin (0·1 μg rennin N/ml) at pH 6·7 and 25°C is examined. 1·0 per cent. nitrogen is rapidly released from whole casein, 3·4 per cent. from second-cycle casein—fraction S, and 6·7 per cent. from κ-casein. None is split from other milk protein fractions under the same conditions.

On treatment with rennin, κ-casein is converted into insoluble para-κ-casein and a soluble fraction containing approximately 23 per cent. of the total nitrogen. Preliminary analyses indicate that this "soluble nitrogen" contains the "glyco-macropeptide" of molecular weight 6000–8000 (cf. Nitschmann, Wissmann, and Henzi 1957). It is therefore suggested that the primary action of rennin on casein is to release this glyco-macropeptide from κ-casein thus destroying the micelle-stabilizing properties of the latter. End-group analyses of κ-casein and para-κ-casein suggest that this process does not involve the rupture of peptide bonds.

I. INTRODUCTION

The mechanism of milk clotting by rennin can be broadly resolved into two stages: (*a*) the enzymic conversion of casein into paracasein, and (*b*) the subsequent coagulation in the presence of calcium ions. There has been much controversy as to which of the components of casein functions as the "protective colloid" of Linderstrøm-Lang (1929). Until very recently most of the evidence has pointed to α-casein as acting in this capacity, and as being the site of primary attack by rennin.

Nitschmann and his co-workers (Alais *et al.* 1953; Nitschmann and Keller 1955) have shown that during the rennet curdling of milk, the amount of non-protein nitrogen (NPN) which is not precipitated by 12 per cent. trichloroacetic acid (TCA) increases markedly before visible clotting occurs. They concluded that the NPN originates from the α-casein, a negligible amount being split from β-casein under the same conditions. The specific splitting reaction, which sets free the small amount of NPN very quickly, was considered to be directly responsible for milk clotting.

Alais (1956) and Nitschmann, Wissmann, and Henzi (1957) have made a study of the chemical and physical characteristics of the NPN split from whole casein by rennin. Approximately 1·5 per cent. NPN (soluble in 12 per cent. TCA) and 4 per cent. NPN (soluble in 2 per cent. TCA) were rapidly split from whole casein by rennin at 25°C. The NPN soluble in 12 per cent. TCA proved to be mainly a "glyco-macropeptide" with a molecular weight of 6000–8000 and a very unusual composition. On precipitating the protein in the reaction mixture by adjustment to pH 4·7, between 4 and 5 per cent. NPN remained in solution; this also consisted of the glyco-macropeptide together with smaller peptides which formed comparatively slowly. These results therefore suggest that the *primary* action of rennin on casein is to set free the glyco-macropeptide.

---

*Biochemistry Department, University of Sydney; present address: Department of Biochemistry, Stanford University, California.

The discovery of κ-casein (Waugh and von Hippel 1956), its isolation, and the demonstration of its micelle-stabilizing properties in the present series throw doubt on the identification of α-casein as the protective colloid. As discussed earlier in this series the so called "α-peak" obtained on electrophoresis of skim milk or whole casein actually represents an α-κ complex. The alteration of the "α-component" of whole acid casein on rennin treatment (Nitschmann and Lehmann 1947) could therefore result from a primary attack on κ-casein and would not necessarily represent a change in α-casein. Also, the protective colloid properties attributed to "α-casein" and its behaviour under the action of rennin could be due to contamination of the α-casein with κ-casein.

In this paper it will be shown that the NPN which is split from whole casein during rennin treatment stems from the κ-casein. An examination of the free α-amino end-groups before and after the action of rennin is also included, as well as some observations on the nature of the material split from κ-casein.

A preliminary report on part of this work has been presented previously (Wake 1957).

II. Materials and Methods

(*a*) *Crystalline Rennin*

At the suggestion of Berridge (personal communication), "Sterren" cheese-making rennet powder (Benger's Ltd., Holmes Chapel, Cheshire, England) was used as starting material for the preparation of crystalline rennin. The first attempt, using essentially the procedure of Berridge and Woodward (1953), without seeding, was unsuccessful. In later experiments the rennin solution was clarified by centrifuging for 90 min at 5°C and 90,000 *g* in a "Spinco" preparative ultracentrifuge, model L (rotor 30; 28,000 r.p.m.), and reprecipitating once more before setting aside for crystallization. The final solution, light straw in colour, failed to yield crystals after 2 days in the refrigerator. On seeding, however, crystals appeared overnight and continued to increase in quantity for several days. Crystals were also obtained on seeding a solution of a freeze-dried preparation from Australian rennet. Approximately 1 g wet crystals were obtained from 500 g of the "Sterren" powder. The rennin was recrystallized by dissolution in a minimum quantity of 0·1M phosphate buffer (0·1M Na_2HPO_4+0·1M KH_2PO_4) at pH 6·8 and 2°C and readjustment to pH 5·4 with 0·1N HCl. Seeding at this stage was not necessary and crystals appeared overnight. However, the yield was rather low. Because of this and the difficulty in redissolving the crystals to prepare stock solutions, the once-crystallized material was further purified by dissolving in phosphate at pH 6·8 and 2°C, adjusting to pH 5·4, and reprecipitating at room temperature by direct saturation with NaCl.

The precipitated material was obtained by centrifuging for 40 min at 5°C and 13,000 r.p.m. in a M.S.E. type 92110 high-speed refrigerated centrifuge using a type S775 rotor, and stored in the moist condition at 2°C in the presence of thymol. On dissolving this material in a minimum quantity of iced water crystallization occurred very readily. The crystalline material had the same form as that described by Berridge (1955).

The once-crystallized and precipitated rennin gave a single peak on electrophoresis in phosphate buffer (Miller and Golder 1950) at pH 6·0, ionic strength 0·1, with a mobility of $-2 \cdot 95 \times 10^{-5}$ cm^2 V^{-1} sec^{-1} at 0·7 per cent. protein concentration. In agreement with Schwander, Zahler, and Nitschmann (1952), the material showed heterogeneity (abnormal spreading) on sedimentation in the pH 6·0 buffer at 2°C. The S_{20} value was approximately 4·4S, which is near the value reported by Schwander, Zahler, and Nitschmann (1952).

(*b*) *NPN Liberation Experiments*

(i) *Total Whey Protein*.—The supernatant, obtained after removal of the casein from the skim milk by the method of von Hippel and Waugh (1955), was used as a source of the total whey protein. It was filtered through Whatman No. 3 paper at 2°C to remove most of the fat, centrifuged at 90,000 *g* and 5°C for 90 min in a "Spinco" preparative ultracentrifuge, and dialysed exhaustively against 0·1M NaCl at 2°C. It was then freeze-dried and stored at 2°C.

(ii) *Experimental Procedure*.—Stock solutions of milk protein fractions were prepared by dissolving the freeze-dried material in 0·1M NaCl and dialysing against a large volume of the same solvent at 2°C overnight. After nitrogen estimations, solutions of required concentration were made up by first adjusting to pH 6·7 with 0·05N HCl or 0·05N NaOH, and then diluting in the correct proportion with 0·1M NaCl. Stock rennin solutions were prepared by dissolving the once-crystallized and reprecipitated material in ice-cold 0·1M NaCl. The nitrogen content of this stock solution was also determined.

20 ml milk protein solution, contained in a 50-ml conical flask, were warmed in a water-bath at 30°C for 20 min. Approximately 0·1 ml rennin solution was pipetted into the protein solution with thorough mixing to give a final concentration of 0·1 μg rennin N/ml. Samples (2 ml) were removed at suitable intervals and pipetted quickly into 4 ml 18 per cent. TCA at room temperature. After mixing, the solution was allowed to stand for 10 min before filtering through a small Whatman No. 42 paper. The nitrogen content of a sample of the clear filtrate was then estimated. NPN values at zero time were obtained from samples taken just prior to the addition of the rennin.

(iii) *Nitrogen Estimations*.—These were obtained by direct Nesslerization of a sample, after digestion under conditions suggested by McKenzie and Wallace (1954) but without the metal catalyst. The procedure used afforded results reproducible to within 5 per cent. over the range 40–150 μg N. Tryptophan (100 μg N) gave 98 per cent. recovery even in the presence of 12 per cent. TCA.

(c) α-*Amino End-group Analysis*

(i) *Preparation of Para-κ-casein.*—Approximately 50 ml 1 per cent. κ-casein in 0·1M NaCl at pH 6·7 was allowed to react with rennin (0·1 μg rennin N/ml final concentration) at 30°C for 30 min. The reaction mixture was cooled in an ice-bath and centrifuged for 50 min at 13,000 r.p.m. in a M.S.E. high-speed refrigerated centrifuge. The clear supernatant was poured off and the sediment treated with water at 80°C for 10 min to inactivate the enzyme. It was then washed with alcohol, ether, and dried in a vacuum over P_2O_5. The supernatant, on adjustment to pH 4·6 with 0·05N HCl, gave only a slight cloudiness. It was heated to 80°C for 10 min, cooled, and readjusted to pH 7 with 0·05N NaOH. It was then freeze-dried. Approximately 400 mg para-κ-casein and 100 mg "soluble nitrogen" (after correcting for NaCl) were obtained.

(ii) *End-group Analysis Procedure.*—Fluorodinitrobenzene (FDNB) (L. Light & Co., England) was used throughout. Most of the dinitrophenyl (DNP)-amino acids were prepared by the methods of Fraenkel-Conrat, Harris, and Levy (1955) or Sanger (1945). 2-DNP-lysine was prepared according to Porter and Sanger (1948). The DNP-derivatives had melting points close to those reported in the literature, and all moved as well-defined spots at the correct rates in the paper chromatographic systems used.

The paper chromatographic systems found most useful were the "toluene"–phosphate, and *tert.*-amyl alcohol systems described by Fraenkel-Conrat, Harris, and Levy (1955). Ethylene chlorhydrin, toluene, and *tert.*-amyl alcohol (laboratory grade) were distilled before use. Solvents used for washing and extracting the DNP-derivatives were also distilled. Peroxide-free ether was used throughout. All other chemicals were A.R. grade.

The DNP-derivatives of κ- and para-κ-casein were prepared as follows: 0·6 g κ-casein were dissolved in 40 ml water and adjusted to pH 9·2 with 0·5N NaOH. 0·6 ml FDNB in 5 ml ether were added with vigorous stirring. The pH was maintained at 9·2 for 2½ hr by the addition of dilute NaOH from a burette. The solution was extracted four times with an equal volume of ether to remove excess FDNB. 1N HCl was added to pH 3·8 and the yellow suspension extracted again with ether to remove dinitrophenol. The precipitate was then centrifuged out, and washed with water, acetone, and finally ether. It was dried in a vacuum over P_2O_5. Para-κ-casein (0·4 g) was dissolved at pH 12 and readjusted to pH 9·2 before reaction with FDNB. This protein aggregates very readily near neutral pH so it was necessary to use the high pH, 9·2, for reaction with FDNB.

The hydrolysis and extraction procedure was carried out as follows: Approximately 20 mg DNP-protein were hydrolysed with 1 ml constant-boiling HCl (glass-distilled) at 105°C in a sealed tube, usually for 16 hr. After cooling it was diluted with 4·7 ml water and centrifuged to remove the brown insoluble humin, which was only formed in any quantity in the case of DNP-κ-casein. The solution was extracted four times with 5 ml ether (only a faint yellow colour was transferred to the ether layer in each case). The combined extracts were evaporated in a vacuum at room temperature. The water layer was evaporated to dryness in a vacuum at 50°C. Both layers were then examined for DNP-amino acids by the chromatographic systems mentioned.

The ether layer could be transferred to the paper with small portions of acetone. The water layer, however, proved rather difficult to handle. This was due to the large amount of ε-DNP-lysine always present. Acetone, containing a trace of concentrated HCl, was generally used to extract the yellow colour. In some cases it was extracted directly from the water layer, before evaporation, with *n*-butanol.

Quantitative estimations of the DNP-amino acids were carried out on the hydrolysates from approximately 40 mg DNP-protein using the procedure of Fraenkel-Conrat, Harris, and Levy (1955). The simplified procedure for the identification and estimation of DNP-arginine described by Wissmann and Nitschmann (1957) was applied to the water layer in each case.

Details of other materials and methods have been given in Parts I and II of this series (McKenzie and Wake 1959*a*, 1959*b*).

III. Results

(*a*) *The Liberation of NPN from Milk Protein Fractions by Rennin*

The results of an investigation of the liberation of NPN (soluble in 12 per cent. TCA) from various milk protein fractions by crystalline rennin are summarized, in Figure 1. The first-cycle casein, second-cycle casein—fractions P and S, and the total whey protein were all prepared from the one milk sample. With first-cycle casein 1·0 per cent. NPN is released within 20 min while none is split from the total whey protein. There is no significant release of NPN with casein from which the κ-component has been removed (second-cycle casein—fraction P) while a marked increase to 3·4 per cent. is evident with second-cycle casein—fraction S. With pure κ-casein approximately 6·7 per cent. NPN is released rapidly. No significant splitting occurs with pure α-casein.

Aggregates form during the action of rennin on the pure κ-casein. On addition of the enzyme to a clear solution of κ-casein the latter begins to appear cloudy. This cloudiness increases in intensity, and the reaction mixture has the appearance of skim milk by the time the maximum value for NPN is reached. These aggregates can be centrifuged out to leave a clear supernatant, containing 23 per cent. of the total nitrogen, which gives no significant precipitate on adjustment to pH 4·6. (The 23 per cent. value could be slightly high due to the trace of β-casein in the κ-casein preparation.) The aggregated material, which will be referred to as para-κ-casein, appeared to move as a single component on paper electrophoresis in veronal buffer at pH 9·0, ionic strength 0·1 (Fig. 2). Extensive adsorption of para-κ-casein makes the identification of traces of other components difficult.

The "soluble nitrogen" (23 per cent.) gives a deep red colour when boiled with Bial's reagent and this is due to the presence of neuraminic acid. It also contains phosphorus (molybdate), and gives a positive sulphuric acid–cysteine test (Dische 1953, 1954) for galactose.

The NPN values obtained at zero time might be of some significance in indicating a small amount of TCA-soluble nitrogen in first-cycle casein and second-cycle casein—fraction S. The zero time values for the various casein fractions were as follows: first-cycle casein, 1·4 per cent.; second-cycle casein—fraction P, 0·6 per cent.; second-cycle casein—fraction S, 3·1 per cent.; κ-casein, 1·2 per cent. A certain zero time value was expected in each case as a result of slight hydrolysis caused by the TCA at room temperature—lower values would almost certainly have been obtained had the TCA been cooled to 0°C before addition of the protein sample. It is possible that there is approximately 0·6 per cent. TCA-soluble nitrogen in first-cycle casein and this remains in the supernatant (second-cycle casein—fraction S) on treatment with 0·25M $CaCl_2$ to give the precipitated second-cycle casein—fraction P. All or most of this TCA-soluble nitrogen is removed during the isolation of pure κ-casein. It is certainly not any of the whey proteins which give a zero time value of only 2·2 per cent.

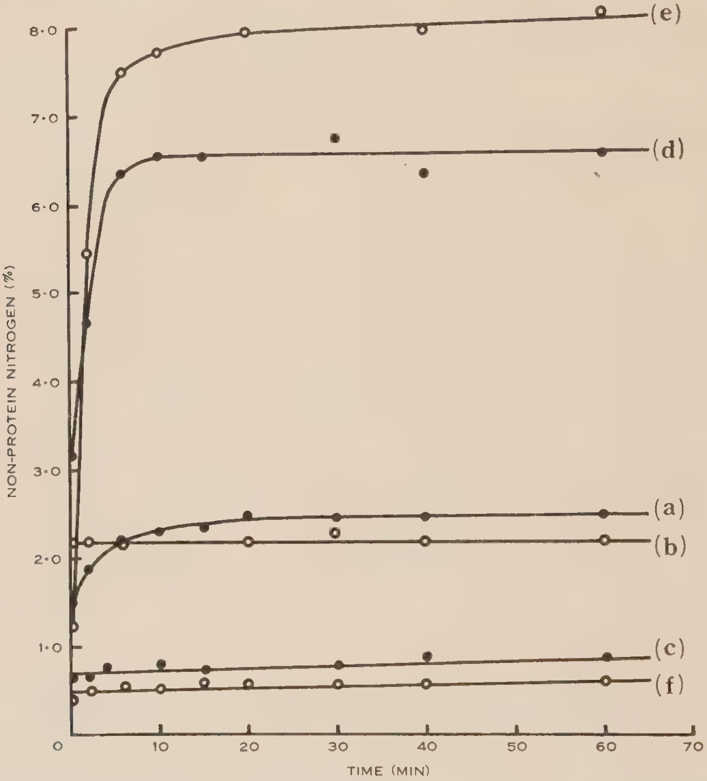


Fig. 1.—Liberation of non-protein nitrogen (soluble in 12 per cent. TCA) from milk protein fractions by rennin. All studies were made at pH 6·7 and 30°C using a preparation containing 0·1 μg rennin N/ml. (*a*) 2 per cent. first-cycle casein; (*b*) 1 per cent. total whey protein; (*c*) 2 per cent. second-cycle casein—fraction P; (*d*) 1 per cent. second-cycle casein—fraction S; (*e*) 1 per cent. κ-casein; (*f*) 2 per cent. α-casein.

(*b*) *α-Amino End-groups in κ-Casein and Para-κ-casein*

Examination of the ether layer, obtained from the hydrolysis of DNP-κ-casein by the two-dimensional toluene–phosphate and *tert*.-amyl alcohol systems indicated the presence of dinitroaniline, dinitrophenol, small amounts of DNP-aspartic and

DNP-glutamic acids, and traces of DNP-serine and DNP-threonine. Also present was an orange-coloured derivative which moved just ahead of threonine in the *tert.*-amyl alcohol system, and in the valine area in the two-dimensional system. Like dinitrophenol, it was decolorized by HCl, and therefore did not represent a DNP-amino acid. It is most likely identical with one of the "orange-coloured artefacts" observed by Redfield and Anfinsen (1956) during their studies on the structure of ribonuclease. No traces of additional DNP-amino acids could be identified in the ether layer with short hydrolysis periods of 2 and 10 hr. It is unlikely that DNP-proline, DNP-glycine, and bis-DNP-cystine would have been *completely* destroyed under these conditions. Certainly the two breakdown derivatives of DNP-proline (Fraenkel-Conrat, Harris, and Levy 1955) were absent. The absence of cysteine from whole casein (Gordon *et al.* 1949) made it unnecessary to check for the presence of this amino acid as an end-group.

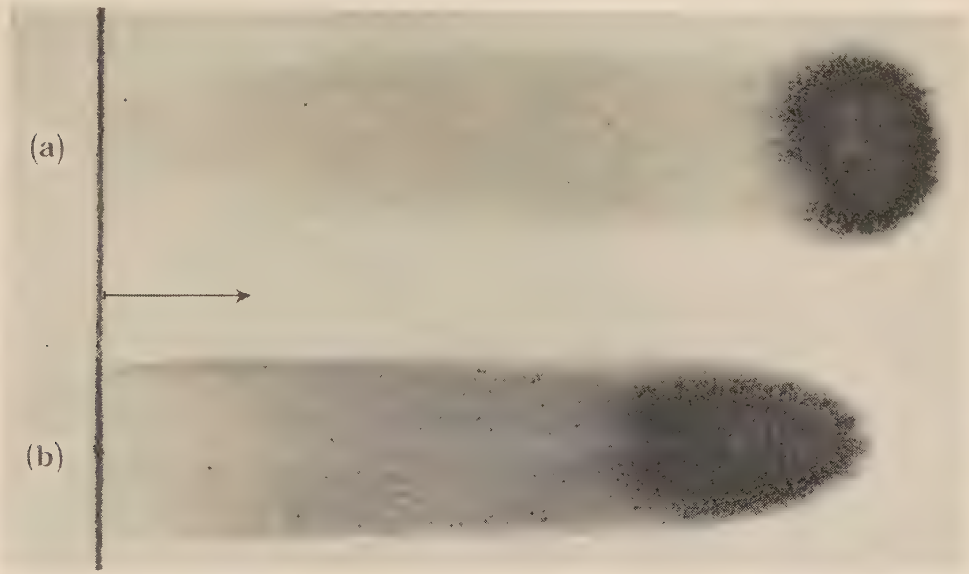


Fig. 2.—Paper electrophoresis patterns of (*a*) κ-casein and (*b*) para-κ-casein in veronal, pH 9·0, ionic strength 0·1, at 2°C, using Whatman No. 1 paper, and run for 14 hr at 140 V.

Examination of the ether layer from DNP-para-κ-casein indicated the presence of dinitroaniline, dinitrophenol, small amounts of DNP-aspartic and DNP-glutamic acids, and traces of DNP-serine, DNP-threonine, bis-DNP-lysine, DNP-leucine or DNP-isoleucine or both, and DNP-phenylalanine. The orange-coloured artefact was again present.

In both cases only DNP-aspartic and DNP-glutamic acids were present in measurable amounts. A quantitative evaluation of these DNP-amino acids was made after chromatographing the ether-soluble derivatives, from a 16-hr hydrolysate, in the *tert.*-amyl alcohol system which gave the best separation of DNP-aspartic and DNP-glutamic acids. The absorption curves of the DNP- derivatives were checked over the range 335–390 mμ and found to be typical of a DNP-amino acid. A breakdown of 30 per cent. for these DNP-amino acids under the hydrolysis conditions used (Porter 1951) was assumed in evaluating the results of this quantitative study as shown in Table 1. Taking 23 per cent. nitrogen as being split from κ-casein, a molecular weight of 18,000 has been assumed for para-κ-casein. It is obvious that DNP-aspartic and DNP-glutamic acids are themselves present only in trace amounts, and there is essentially no difference between κ-casein and para-κ-casein in this respect.

The only DNP-amino acids of significance which could have been present in the water layer were ϵ-DNP-lysine, DNP-arginine, and bis-DNP-histidine. ϵ-DNP-lysine, is very frequently present in large excess and this renders the identification and estimation of the other two very difficult. No trace of the bis-DNP-histidine could be detected in the ether layer, even after exhaustive extraction with ether. It was, therefore, unlikely to appear in significant quantities in the water layer, and could not be detected in any of the chromatographic systems used. The phosphate system proved most useful as bis-DNP-histidine moved very slowly in comparison with the others. DNP-arginine moves rather close to ϵ-DNP-lysine in the chromatographic systems used and cannot be separated from the large excess of the latter—an attempt to use the benzene system of Mellon, Korn, and Hoover (1953) resulted in considerable tailing of the spots. However, no DNP-arginine could be detected on chromatograms of the water layers on spraying with Sakaguchi's reagent.

TABLE 1

NUMBER OF FREE α-AMINO END-GROUPS DETECTED IN κ-CASEIN AND PARA-κ-CASEIN BY THE FDNB METHOD

End-group	DNP-κ-casein		DNP-para-κ-casein	
	Mole/10^5g	Mole/Mole	Mole/10^5g	Mole/Mole
Aspartic acid	0·13	*c.* 0·03	0·16	*c.* 0·03
Glutamic acid	0·12	*c.* 0·03	0·13	*c.* 0·02

Because of the presence of arginine as a free α-amino end-group in both α- and β-casein (Mellon, Korn, and Hoover 1953; Wissmann and Nitschmann 1957) and the difficulty in detecting and estimating it by the procedures described above, the simplified procedure of Wissmann and Nitschmann was applied once to the water layer in an attempt to clarify the position. The results indicated the presence of 0·6 and 0·5 arginine α-amino end-groups per 10^5 g DNP-κ-casein and DNP-para-κ-casein respectively. Interfering factors, such as the incomplete removal of free arginine, could give rise to a false estimate of DNP-arginine by this method, and it cannot be said with certainty that κ-casein and para-κ-casein contain free α-amino arginine. It is obvious, however, that there is no significant difference between κ-casein and para-κ-casein in this respect. It is interesting to note that Lea and Hannan (1950) and Schwartz and Lea (1952) reported only trace amounts of glutamyl, lysyl, phenylalanyl, and valyl α-amino end-groups in whole casein. Terminal arginine residues were not found to be present and the results contrast with those of Mellon, Korn and Hoover (1953) and Wissmann and Nitschmann (1957). Seno, Murai, and Shimura (1955), during a study of the lysyl peptides in α-casein by the FDNB technique, could not detect any terminal arginine residues in this component.

IV. Discussion

The results on the liberation of NPN from various milk protein fractions clearly support the conclusion that κ-casein is the site of primary attack when rennin acts on whole casein (Waugh and von Hippel 1956). They are also in accordance with the assumption that the specific splitting reaction, which sets free a small part of NPN very quickly, is directly responsible for milk clotting (Alais *et al.* 1953; Nitschmann and Keller 1955). The NPN/time curves obtained by the latter workers for "α-casein" prepared by the urea method of Hipp *et al.* (1952) can be explained on the basis of contamination with κ-casein.

The release of 1·0 per cent. NPN from first-cycle casein and 6·7 per cent. NPN from κ-casein indicates the presence of approximately 15 per cent. of the latter component in whole casein (cf. Waugh and von Hippel 1956). κ-Casein on rennin treatment is rapidly converted into an insoluble fraction, referred to here as para-κ-casein, and a soluble fraction which accounts for approximately 23 per cent. of the total nitrogen.

The difference between the 6·7 per cent. NPN value obtained when the protein is precipitated with 12 per cent. TCA and the 23 per cent. "soluble nitrogen" remaining in solution when the para-κ-casein is removed by centrifugation suggests that the "soluble nitrogen" is not a single component. However, Alais (1956) has shown that the maximum NPN value (soluble in 12 per cent. TCA) obtained when rennin acts on whole casein depends largely upon the method of adding the TCA to the reaction mixture, and it is almost certain that 12 per cent. TCA causes precipitation of an appreciable portion of the glyco-macropeptide described by him.

Nitschmann, Wissmann, and Henzi (1957) found that 4–5 per cent. of the nitrogen remained in solution when rennin-treated whole casein was precipitated at pH 4·7. Assuming this nitrogen is derived solely from κ-casein, present to the extent of 15 per cent. in whole casein, a value of 27–34 per cent. NPN would be expected on treating κ-casein with rennin. Nitschmann and his co-workers have shown that this material consists of the glyco-macropeptide as well as other smaller peptides which form only very slowly during rennin action in comparison with the glyco-macropeptide. It is therefore possible that the 23 per cent. "soluble nitrogen" split from κ-casein consists largely of the glyco-macropeptide or a product similar to it. A molecular weight of 26,000 for κ-casein (see Part III, McKenzie and Wake 1959*c*) would result in a molecular weight of 8000 for the soluble nitrogen if it were mainly a single component. This is essentially the same as the value of 6000–8000 obtained by Nitschmann *et al.* for the glyco-macropeptide. Certainly the soluble nitrogen split from κ-casein contains this material. Galactose and neuraminic acid, as well as phosphorus, have been found in both. The large amount of humin formed on hydrolysing DNP-κ-casein with HCl is probably derived from neuraminic acid which is known to give such products on acid treatment (Blix 1936). The fact that this effect was not pronounced with DNP-para-κ-casein indicates that most of the neuraminic acid, if not all, is split from κ-casein by rennin and is found in the soluble fraction.

Para-κ-casein moves at a slower rate, when subjected to paper electrophoresis at pH 9, than does κ-casein, and this could be due to the release of an "acidic" macropeptide of the composition indicated by Nitschmann, Wissmann, and Henzi (1957). A comparatively simple procedure for the isolation of κ-casein, completely free from β-casein, has been developed, and it is intended to use this material to investigate more fully the nature of the soluble nitrogen split off by rennin.

It is proposed that the specific action of rennin on κ-casein at neutral pH is to release a glyco-macropeptide of rather unusual composition with the consequent formation of insoluble para-κ-casein. Does the enzyme rupture some specific peptide bond and thus set free a part of a polypeptide chain in κ-casein as the glyco-macropeptide? If this were the mechanism of rennin action then a new α-amino end-group would be expected to appear in either para-κ-casein or the glyco-macropeptide. However, the observations made here indicate essentially no difference between κ-casein and para-κ-casein with respect to their content of α-amino end-groups, and in addition, Nitschmann, Wissmann, and Henzi (1957) could detect no free α-amino end-groups in the glyco-macropeptide. These results suggest that the conversion of κ-casein to para-κ-casein does not rest in the ability of rennin to split peptide bonds. This result was unexpected in view of the observations of Wissmann and Nitschmann (1957) who reported the appearance of phenylalanyl α-amino end-groups to the extent of 1·8 residues/10^5 g protein in rennin-treated "α-casein", presumably contaminated with κ-casein. Their observations suggested that "the primary reaction in the rennet curdling of milk is a specific limited proteolysis". In a recent paper by Fish (1957) it has been shown that rennin has a proteolytic activity similar to that of pepsin. In the B chain of insulin it splits the following five bonds: Leu–Val, Leu–Tyr, Tyr–Leu, Phe–Phe, and Phe–Tyr. The optimum effect is at pH 4. It is possible that the appearance of the phenylalanyl α-amino end-group reported by Wissmann and Nitschmann was due to a similar proteolytic activity involving phenylalanine residues. This would have been enhanced on adjustment to pH 4·6 where the casein was precipitated.

It is generally considered that calcium is needed for the clotting of paracasein, and many workers are of the opinion that calcium acts as a bridge between different paracasein "molecules" (see Higgins and Fraser 1954). The exact mechanism of the clotting stage and the role of calcium await elucidation.

V. ACKNOWLEDGMENTS

Grateful acknowledgment is due to Dr. H. A. McKenzie and Mr. M. B. Smith for their advice and assistance during the course of this work. Thanks are also due to Dr. N. J. Berridge who supplied the seeding crystals of rennin, and to Dr. K. Knox who carried out the preliminary carbohydrate analyses. Other acknowledgments are given in Part I.

VI. References

Alais, C. (1956).—14th Int. Dairy Congr., Rome. Vol. 2. Pt. 2. p. 823.

Alais, C., Mocquot, G., Nitschmann, H., and Zahler, P. (1953).—*Helv. Chim. Acta* **36**: 1955.

Berridge, N. J. (1955).—*Milchwissenschaft* **10**: 195.

Berridge, N. J. and Woodward, C. (1953).—*J. Dairy Res.* **20**: 255.

Blix, G. (1936).—*Hoppe-Seyl. Z.* **240**: 43.

Dische, Z. (1953).—*J. Biol. Chem.* **204**: 983.

Dische, Z. (1954).—In "Methods of Biochemical Analysis". Vol. 2. p. 313. (Ed. D. Glick.) (Interscience Publishers, Inc.: New York.)

Fish, J. C. (1957).—*Nature* **180**: 345.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955).—In "Methods of Biochemical Analysis". Vol. 2. p. 359. (Ed. D. Glick.) (Interscience Publishers, Inc.: New York.)

Gordon, W. G., Semmett, W. F., Cable, R. S., and Morris, W. (1949).—*J. Amer. Chem. Soc.* **71**: 3293.

Higgins, H. G., and Fraser, D. (1954).—*Aust. J. Biol. Sci.* **7**: 85.

Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L. (1952).—*J. Dairy Sci.* **35**: 272.

Hippel, P. H. von, and Waugh, D. F. (1955).—*J. Amer. Chem. Soc.* **77**: 4311.

Lea, C. H., and Hannan, R. S. (1950).—*Biochim. Biophys. Acta* **4**: 518.

Linderstrøm-Lang, K., and Kodama, S. (1925).—*C. R. Lab. Carlsberg* (*Ser. Chem.*) **16**(1): 1.

McKenzie, H. A., and Wake, R. G. (1959*a*).—*Aust. J. Chem.* **12** (4) (in press).

McKenzie, H. A., and Wake, R. G. (1959*b*).—*Aust. J. Chem.* **12** (4) (in press).

McKenzie, H. A., and Wake, R. G. (1959*c*).—*Aust. J. Chem.* **12** (4) (in press).

McKenzie, H. A., and Wallace, H. S. (1954).—*Aust. J. Chem.* **7**: 55.

Mellon, E. F., Korn, A. H., and Hoover, S. R. (1953).—*J. Amer. Chem. Soc.* **75**: 1675.

Miller, G. L., and Golder, R. H. (1950).—*Arch. Biochem. Biophys.* **29**: 422.

Nitschmann, H., and Keller, W. (1955).—*Helv. Chim. Acta* **38**: 942.

Nitschmann, H., and Lehmann, W. (1947).—*Helv. Chim. Acta* **30**: 804.

Nitschmann, H., Wissmann, H., and Henzi, R. (1957).—*Chimia* **3**: 76.

Porter, R. R. (1951).—*Meth. Med. Res.* **3**: 256.

Porter, R. R., and Sanger, F. (1948).—*Biochem. J.* **42**: 287.

Redfield, R. R., and Anfinsen, C. B. (1956).—*J. Biol. Chem.* **221**: 385.

Schwander, H., Zahler, P., and Nitschmann, H. (1952).—*Helv. Chim. Acta* **35**: 553.

Schwartz, H. M., and Lea, C. H. (1952).—*Biochem. J.* **50**: 713.

Seno, N., Murai, K., and Shimura, K. (1955).—*J. Biochem.* **42**: 699.

Wake, R. G. (1957).—*Aust. J. Sci.* **20**: 147.

Waugh, D. F., and Hippel, P. H. von (1956).—*J. Amer. Chem. Soc.* **78**: 4576.

Wissmann, H., and Nitschmann, H. (1957).—*Helv. Chim. Acta* **40**: 356.

STUDIES ON OXIDIZED WOOL

III. ION-EXCHANGE AND ACID-UPTAKE CHARACTERISTICS

By E. O. P. Thompson* and I. J. O'Donnell*

[*Manuscript received May* 1, 1959]

Summary

The acid-uptake and ion-exchange characteristics of wool oxidized with peracetic acid have been re-examined. It has been shown that oxidized wool is capable of ion exchange although the ion-exchange properties are similar to those of carboxylic acid cation-exchange resins and unlike those of sulphonic acid cation-exchange resins. Oxidized wool was successfully converted to the ammonium "salt" and the ammonium ions exchanged with barium ions.

Oxidized wool takes up very little acid even at pH 0·45, which is evidence that the sulphonic acid groups are largely ionized at this pH. It will, however, take up 740 μ-equiv. of alkali per g of dry oxidized wool on titration to pH 5·7 and it is concluded that in water-washed oxidized wool the carboxyl groups are substantially unionized whereas sulphonic acid groups are ionized and internally compensated by the ionized basic groups.

I. Introduction

The properties of oxidized wool prepared by treatment of wool with 1·6 per cent. aqueous peracetic acid were studied by Alexander, Fox, and Hudson (1951). It did not liberate acid when treated with potassium chloride, and after equilibration with ammonium chloride no cation exchange occurred when it was subsequently treated with potassium chloride. If strongly acid sulphonic acid groups were present in oxidized wool it would be expected that they would allow ion exchange to occur under the above conditions. To explain this behaviour Alexander, Fox, and Hudson postulated that in oxidized wool there were no sulphonic acid groups but that intramolecular cyclization occurred to form a sulphonimide grouping (I) which would hydrolyse directly to cysteic acid. However, on treatment with dilute ammonia it was postulated that a sulphonamide type of grouping (II) was formed which would be hydrolysed to cysteic acid only with difficulty.

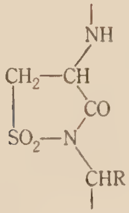
The fact that other oxidized proteins such as oxidized insulin (Sanger 1949*a*, 1949*b*) were known to contain sulphonic acid groups suggested that the explanation of Alexander, Fox, and Hudson is incorrect. Weston (1955) studied the infra-red spectrum of oxidized wool and demonstrated conclusively that $-SO_3^-$ or SO_3H groups were present. Earland and Knight (1955) explained the absence of the expected ion-exchange properties on the assumption that the $-SO_3^-$ groups were bound in "salt linkage" to the basic groups. However, if this is the case there should be excess carboxyl groups capable of ion exchange. Earland and Knight

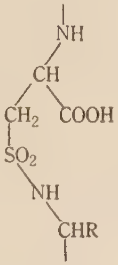
---

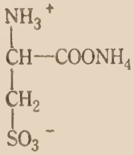
*Division of Protein Chemistry (formerly Biochemistry Unit), C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

reported that α-keratose, the acid-precipitable fraction of the alkali-soluble constituents of oxidized wool, did not form an ammonium salt, a fact which, if true, is not explicable on their theory, since the carboxyl groups should form an ammonium salt as is the case with cysteic acid (III).

The experiments reported in this paper on the ion-exchange and acid-uptake properties of oxidized wool support the view that $-SO_3^-$ groups are present. The ion-exchange properties are those of carboxylic acid groups and are similar to those of the carboxylic acid type of cation-exchange resins, the behaviour of which is very different from that of the sulphonic acid type of ion-exchange resin (Kunin and Barry 1949).


(I)


(II)


(III)

II. Materials and Methods

The Merino 64's wool used was that described in Part I of the series (Thompson and O'Donnell 1959). Wool oxidized for 24 hr with 1·6 per cent. aqueous peracetic acid as described by O'Donnell and Thompson (1959) was used in all experiments.

Measurements of pH were made with a glass electrode standardized at pH 4 with 0·05M potassium hydrogen phthalate. Automatic titrations were made with a pH-stat of the Jacobsen and Léonis (1951) type with a Radiometer pH-meter coupled with an "Agla" micrometer syringe and recorder (Ole Dich, Copenhagen).

III. Experimental

All values refer to wool or oxidized wool of zero moisture content.

(a) *Ion-exchange and Acid-uptake Properties of Oxidized Wool*

Samples of normal and oxidized wools were washed in a large excess of 0·001N hydrochloric acid for 24 hr and then washed free of acid, dried, and equilibrated in air. The acid-uptake and ion-exchange properties were then studied in three ways:

(1) Samples (1 g) were shaken (using a wrist-action shaker) for at least 30 min with 20 ml 1N potassium chloride solution containing either 1N or 0·05N hydrochloric acid. These conditions are more than adequate for the attainment of equilibrium (Alexander *et al.* 1951). 1-ml samples of solution were then back-titrated with standard 0·1N alkali (Alexander, Fox, and Hudson 1951). The experiments were carried out in replicate.

(2) Samples (1 g) of oxidized wool were shaken successively (using a wrist-action shaker) for 15–30 min with 30-ml aliquots of 1N potassium chloride at pH 6·0 and the pH was measured at the completion of each shaking. Another sample (0·5 g) of the oxidized wool was packed in a column 1·0 cm in diameter and eluted successively with 100-ml portions of 1N potassium chloride (pH 5·8). The pH of the eluate was measured and the eluate then titrated to pH 5·8–6·0 with standard alkali.

(3) Samples (0·5 g) of oxidized wool were titrated, with stirring, in 100 ml 1N potassium chloride to pH 5·7 with a pH-stat and the total amount of alkali consumed was determined.

(b) *Equilibration of Oxidized Wool with Ammonium Chloride Solution*

Oxidized wool (0·5 g) was successively equilibrated five times with 20 ml 1N ammonium chloride (pH 4·7) solution. Usually the mixture was left standing 2–16 hr before changing the aqueous medium. After the fifth equilibration the oxidized wool was carefully washed with water (5×20 ml) till free from chloride and ammonium ions (Nessler's reagent). The wool was then eluted with 20 ml 1N barium acetate solution at pH 6·5 and after 5 min the ammonium ions liberated into the supernatant were estimated after distillation from an alkaline solution into boric acid and titration with 0·02N acid.

(c) *Equilibration of Oxidized Wool with Ammonium Phosphate Buffer*

Oxidized wool (1 g) was equilibrated with 20 ml 0·28M ammonium phosphate buffer of pH 6·5 ($NH_4H_2PO_4$ 13·8 g and $(NH_4)_2HPO_4$ 10·6 g in 1l. of solution). After standing overnight the oxidized wool was swollen and some material had dissolved (cf. O'Donnell and Thompson 1959). After carefully washing with distilled water the filtrate gave a negative test for phosphate by the benzidine–molybdate reagent (Vogel 1945) and only a very slight positive test for ammonium ion by Nessler's reagent. (That phosphate was almost entirely absent was shown by the fact that tap water, containing only traces of phosphate (0·02 mg/l), gave a positive test.) During water-washing, the fibres swelled to a gelatinous material that could only be filtered off slowly on a Buchner funnel using Whatman No. 54 filter paper. A sample of oxidized wool which had previously been heated to 105°C for dry weight determination did not swell to the same extent. The residual oxidized fibre (a correction was made for absorbed water) was eluted with 10 ml 1N barium acetate solution (pH 6·5) and after 5 min the ammonium ions liberated into the supernatant were estimated as described in the previous section.

(d) *Demonstration of the Formation of an Ammonium Salt by α-Keratose*

A solution of α-keratose, prepared by extraction at pH 11 of performic acid-oxidized wool (O'Donnell and Thompson 1959), was dialysed overnight in a rocking dialyser against 0·1M ammonia solution. Aliquots (2 ml) of the solution of α-keratose were evaporated in a vacuum over sulphuric acid and caustic soda. Equal-sized aliquots were similarly evaporated for 3 hr and then 2 ml 0·2M potassium carbonate were added, followed by evaporation in a vacuum overnight to remove any bound ammonia. The nitrogen contents of the total dried residues were than determined by the Kjeldahl procedure (Chibnall, Rees, and Williams 1943).

IV. Results and Discussion

(a) *Ion Exchange and Acid Uptake of Wool Oxidized with Peracetic Acid*

(i) *Washing of Oxidized Wool with Potassium Chloride Solution.*—Oxidized wool (5 g) on shaking for 2 hr with 200 ml water (pH 5·5) caused the pH to fall to 5·2; hence the washed oxidized wool contains virtually no free acid. However, on shaking 1-g samples with 30 ml 1N potassium chloride solution (adjusted to pH 6·0) the pH fell to 3·3 and after six successive shakings with fresh potassium chloride the pH was only 3·65. Two subsequent shakings with 200 ml 1N potassium chloride raised the pH to 4·0. It can be seen that it would require many shakings to raise the pH to the value for unbuffered potassium chloride, i.e. approximately 5·8. In an effort to equilibrate the oxidized wool to pH 5·8 with 1N potassium chloride solution it was washed in a column. By slowly passing 1300 ml 1N potassium chloride solution through the column (containing 0·5 g oxidized wool) the pH of the effluent slowly rose from 3·3, that of the last 100 ml being 4·6. The amount of alkali required to titrate the total 1300 ml of effluent to pH 5·8 was equivalent to 407 μ-equiv/g oxidized wool. It can be seen that it is impracticable to wash oxidized wool with unbuffered 1N potassium chloride until the pH of the potassium chloride is unchanged by passage through the wool.

Alexander, Fox, and Hudson (1951) state that they first equilibrated their oxidized wool samples (mainly partially oxidized wools) at pH 6·0 by successive shakings with potassium chloride solution before determining the acid uptake. No further details were given and it is not clear whether, in fact, a pH of 6·0 was reached.

(ii) *Uptake of Acid.*—With 1N hydrochloric acid in 1N potassium chloride it was found that normal wool took up 810 μ-equiv. of acid per g of wool, which is in good agreement with literature values (cf. Alexander and Hudson 1954). However, oxidized wool under the same conditions took up only 160 μ-equiv. of acid per g of original wool, allowing for the 3·5 per cent. increase in weight of wool on oxidation. These results which were obtained at pH 0·45 were intended to show the difference in acid uptake of normal and oxidized wool assuming any sulphonic acid groups in oxidized wool would not be ionized at these low pH values. It appears that oxidized wool has an even lower acid uptake than normal wool and, assuming that sulphonic acid groups are present, this could be due to the fact that:

(1) An acid-rich fraction dissolves on oxidation of wool with aqueous peracetic acid. However, even if the 1 per cent. of dissolved material (Thompson and O'Donnell 1959) were pure cysteic acid it would not account for this discrepancy.

(2) Some of the oxidized wool protein dissolves in the potassium chloride–hydrochloric acid solution. This factor cannot be a major one since nitrogen estimations showed that only 4 per cent. of the wool dissolved.

(3) The sulphonic acid groups present in oxidized wool are still ionized almost completely at pH 0·45 and the carboxyl groups are practically unionized in water-washed wool at the beginning of the acid-uptake experiments.

The acid uptake of oxidized wool in 1N hydrochloric acid–1N potassium chloride was compared with that in 0·05N hydrochloric acid–1N potassium chloride. The acid uptake was small in both cases, being 66 μ-equiv/g for the 0·05N hydrochloric acid and 154 μ-equiv/g for the 1N hydrochloric acid.

With wool partially oxidized (1 hr) with peracetic acid (0·25N) Alexander, Fox, and Hudson (1951) found a maximum acid-combining capacity similar to that for untreated wool. Treatment with alkali gave an increase in acid-combining capacity. If, as discussed earlier, Alexander, Fox, and Hudson had, in fact, equilibrated their samples to pH 6·0 before titration with acid the carboxyl groups would have been ionized. They would then have back-titrated these ionized carboxyl groups and consumed acid equivalent to the alkali taken up during our equilibration (to pH 5·7) with the pH-stat. We are unable to explain their finding that partially oxidized wools on treatment with alkali had an acid-combining capacity in excess of that of untreated wool.

(iii) *Titration of Oxidized Wool with a pH-stat with Sodium Hydroxide.*—From the acid-uptake experiments and the fall in pH on washing wool with potassium chloride solution it must be concluded that factor (3) above is correct. Titration of the oxidized wool to pH 5·7 with the pH-stat showed that the wool took up 740 μ-equiv. alkali per g of oxidized wool over a period of 21 hr, less than 10 μ-equiv. being taken up over the last 6 hr. The wool which had been exhaustively washed on a column with potassium chloride when similarly titrated to pH 5·7 took up, over the course of 3 hr, an additional 236 μ-equiv. of alkali (per g oxidized wool) making a total alkali uptake of 643 (236+407) μ-equiv. (see Section IV(*a*)) alkali per g of oxidized wool. This latter value is not as accurate a value for the alkali consumption as that obtained by direct titration because of difficulties in the accurate titration of large volumes of the 1N potassium chloride from the column in which the oxidized wool was washed.

Since the uptake of hydrochloric acid by oxidized wool in the pH range 1·43–0·45 is only 66–154 μ-equiv. whereas the alkali consumed in titrating the oxidized wool to pH 5·7 is 740 μ-equiv/g, it is concluded that the carboxyl groups in oxidized wool are substantially unionized while the sulphonic acid groups are ionized.

It was further observed that on washing oxidized wool with water at pH 5·5 no protein dissolved but when 1N potassium chloride was present and the pH kept at pH 5·7 a substantial amount of wool (14 per cent.) dissolved. Ionization of the carboxyl groups, which is possible in the presence of a counterion (K^+), must be a prerequisite for solution of the oxidized wool in aqueous media.

(*b*) *Ion-exchange Characteristics of Oxidized Wool*

When oxidized wool was treated with ammonium chloride solution of pH 4·7 the pH fell within 2 min to pH 3·4 and only fell by a further 0·05 units overnight. Repeated treatment with fresh 1N ammonium chloride solution did not affect this behaviour which is similar to that reported for oxidized wool with solutions of potassium chloride. After five successive treatments the oxidized wool was washed with water until it was free from chloride ions and eluted with barium acetate solution when it liberated its bound ammonium ions. Estimation of this displaced ammonia showed that only 79 μ-equiv/g of oxidized wool were present.

If $-SO_3^-H^+$ groups capable of ion exchange were present with characteristics similar to those of a sulphonic acid type of cation-exchange resin (e.g. sulphonated polystyrene) ammonium ions equivalent to the $-SO_3^-H^+$ groups should have been bound (731 μ-equiv/g of oxidized wool (Table 1) and more if sulphinic acids with a pK value of about 1–2 are present) as pointed out by Alexander, Fox, and Hudson (1951). However, if the $-SO_3^-$ groups are internally compensated by the ionized basic groups in the oxidized wool they are not available for ion exchange and only the excess unionized carboxyl groups would be capable of exchanging ions. Under these conditions the binding of ammonium ions from an ammonium chloride solution by oxidized wool would be expected to be small since the weak carboxylic acid groups will not exhibit ion-exchange behaviour at low pH values.

TABLE 1

ACIDIC AND BASIC GROUPS IN MERINO 64'S WOOL

Values in μ-equiv/g dry wool or oxidized wool

Group	Normal Wool		Wool Oxidized with Aqueous Peractic Acid
	Simmonds (1956)	Corfield and Robson (1955)	Corfield, Robson, and Skinner (1958)
Aspartic acid	476–537	511	511
Glutamic acid	1031	990	953
Cysteic acid	—	—	729 $\left.\begin{array}{l}722\dagger\\742*\dagger\end{array}\right\}$ 732
α-Carboxyl groups	10§	10§	(10)¶
Amide groups	745‡	786	1047 (720)¶
Total acidic groups	772–833	725	1483
Arginine	520	557	538
Histidine	53	74	63
Lysine	197	229	225
α-Amino groups	28‖	28‖	(28)¶
Total basic groups	798	888	854

* This value increases to 838 on further oxidation with performic acid (Thompson and O'Donnell 1959).

† Thompson and O'Donnell (1959).

‡ Leach and Parkhill (1956).

§ Bradbury (1958).

‖ Thompson (1959).

¶ Values for unoxidized wool.

The oxidized wool behaves like a carboxyl (weak acid) type of cation-exchange resin (e.g. "Amberlite IRC-50") the properties of which have been discussed by

Kunin and Barry (1949). Thus, in the hydrogen cycle the –COOH groups are capable only of adsorbing cations present as the hydroxides, carbonates, or salts whose acids are not sufficiently strong to cause the pH to fall below 4–5 during the experiment. With salts of strong acids, as the pH falls to below 4 only hydrogen ions are held by the carboxyl groups due to the marked affinity of carboxyl groups for these ions. To lower the pH of 20 ml of 1N ammonium chloride solution from 4·7 to 3·5 requires only 6 μ-equiv. of hydrochloric acid. Thus, only a small proportion of the carboxyl groups in oxidized wool (Table 1) would bind ammonium ions from ammonium chloride solution before the pH fell to a value at which no uptake of ammonium ions by carboxyl groups would be expected. We can therefore account for the failure of Alexander, Fox, and Hudson (1951) to detect ion exchange with ammonium chloride or potassium chloride solutions. The 79 μ-equiv/g detected in our experiments represents the small amount of ammonium ion taken up before the pH fell to a value at which exchange ceased.

When an ammonium buffer of pH 6·5 was substituted for ammonium chloride solution the pH fell to 6·35 after standing overnight. On washing free of ammonium and phosphate ions the residual wool was eluted with barium acetate solution and ammonium ions equivalent to 476 and 532 (mean 504) μ-equiv/g of oxidized wool were liberated. When a carboxylic acid resin (1 ml wet "Amberlite IRC–50") in the acid form was substituted for oxidized wool in this experiment similar behaviour was observed. The pH fell to 6 in about 7 hr and after standing overnight with fresh buffer the pH was 6·35. The quantitative figures for bound ammonium ion in the exchange experiment with oxidized wool will be low for the following reasons. Firstly, if the time required for equilibration is similar to the 7 days required by carboxylic resins (Kunin and Barry 1949) the exchange cannot be continued for the necessary time since the oxidized wool is dissolving. Thus O'Donnell and Thompson (1959) found 34 per cent. of the nitrogen was extracted in 24 hr at pH 6·5 in the absence of salt. Secondly, being the "salt" of a weak acid ammonium ions will be lost by hydrolysis during washing of the ammonium "salt" to free it of excess buffer. However, it is clear that there is in oxidized wool a large number of carboxylic acid groups capable of ion exchange. Further, Hale and Reichenberg (1949) have shown that there is a spread of pK values in carboxylic ion-exchange resins and that the capacity for cations (other than hydrogen ion) increases with cation concentration and pH. The resin does not exhibit its full capacity below pH 8–9. With oxidized wool it was found that with a 10-fold increase in ammonium ion concentration (2·8M ammonium phosphate at pH 6·5 and equilibrating for 64 hr) the bound ammonium ions displaced by the barium ion increased to 693 μ-equiv/g oxidized wool. This value agrees well with the alkali-uptake value (740 μ-equiv/g). The increased recovery with the more concentrated buffer is probably due to the decreased extraction of wool proteins in the presence of salt (O'Donnell and Thompson 1959).

The results of both the acid-uptake and ion-exchange experiments therefore support the conclusions of Weston (1955) and Earland and Knight (1955) that oxidized wool contains $-SO_3^-$ groups but these are internally compensated by the basic groups in the wool. The pK of these sulphonic acid groups must be near zero since they are still ionized at pH 0·45. The pK of the sulphonic acid groups in cysteic acid is 1·3 (Andrews and Schmidt 1927) but when involved in "salt-linkage" the pK values for acidic groups have been lower than normal (Lemin and Vickerstaff 1946).

In our previous work it was shown that 742 μmoles of cysteic acid are present per g of oxidized wool (Table 1) which increases to 838 on further oxidation with performic acid. The state of these 96 μmoles of partially oxidized cystine is not known but it could be in a partially or wholly acidic form, e.g. $-SO_2H$, so that the maximum possible number of "sulpho" acids in wool oxidized with peracetic acid is 838 μmoles/g of oxidized wool.

There is general agreement that the isoelectric point of wool is less than 7, so that at neutral pH the number of ionized negative groups exceeds the number of ionized positive groups. The amino acid composition of normal wool given in Table 1 compares the analytical values of Simmonds (1956) using the value for amide nitrogen of Leach and Parkhill (1956) with those of Corfield and Robson (1955). The former show a small excess of acidic residues particularly if a correction is made for the small proportion of histidine residues ionized at pH 7 (pK approx. 6) whereas the latter show an excess of basic residues. Hence the values of Simmonds combined with the amide values of Leach and Parkhill are likely to be the best at present available.

Treatment of wool with peracetic acid would not be expected to increase the content of amide groups; rather a decrease would be expected. The very high value of 1047 μ-equiv/g reported by Corfield, Robson, and Skinner (1958) may be due to increased ammonia liberation on treatment with acid in the presence of sulphonic acid groups (which catalyse hydrolysis reactions) in the oxidized fibre (Steinhardt and Fugitt 1942; Sanger 1949*b*). For this reason, and bearing in mind the arbitrary nature of the methods for amide estimation, we have chosen the value of 720 μ-equiv/g (the value for whole wool corrected for the increase in weight during oxidation).

From Table 1 the basic groups of oxidized wool total 854 μ-equiv/g, the free carboxyl groups 754 μ-equiv/g, and the cysteic acid groups 742 μ-equiv/g (838 μ-equiv/g if all possible "sulpho" acid groups are included).

Titration of oxidized wool with the pH-stat indicated the content of unionized carboxyl groups to be 740 μ-equiv/g and ionized carboxyl groups may contribute to the 154 μ-equiv/g of acid required to titrate the wool to pH 0·45. The value is therefore in good agreement with the value of 754 μ-equiv/g for free carboxyl groups quoted above. Hence in washed oxidized wool the 854 μ-equiv. of basic groups per g must be internally compensated by the 742 μ-equiv. of cysteic acid groups together with any sulpho acid groups—Cavallino *et al.* (1958) estimate a value of 2·16 for the pK of ethane sulphinic acid and Lavine (1936) a value of 2·1 for cystine sulphinic acid—and perhaps a small number of ionized carboxyl groups.

The calculations in this paper suffer from an inherent degree of uncertainty due to variation in the literature values for the amino acid composition of wool. Further work is obviously necessary to fix these values more accurately.

Earland and Knight (1955) claimed that whereas cysteic acid formed a monoammonium salt, α-keratose did not. We have confirmed their first finding and showed that treatment of the cysteic acid salt with potassium carbonate to remove ammonium ions causes the nitrogen content to revert to the value for cysteic acid. However, after dialysis of a solution of α-keratose to equilibrium with 0·1N ammonia and evaporation of excess ammonia in a vacuum the Kjeldahl titres were 10·04, 10·10, and 10·07 ml of 0·02N acid. After removal of ammonium ions with potassium carbonate the titres for equal quantities of the α-keratose were 9·54, 9·64, and 9·56 ml. The difference of 0·49 ± 0·03 (S.E.) in the mean values of 10·07 and 9·58 is significant at the 0·1 per cent. probability level. The α-keratose used in these experiments contained an excess of acid side-chains of about 490 μ-equiv/g (Table 2). It can be calculated that if an ammonium ion is bound at each of the excess carboxyl groups the nitrogen content of 16 per cent. for α-keratose would be increased by 4·3–4·8 per cent. depending on the amide nitrogen content of the preparation. This corresponds closely with the 5·1 per cent. increase reported above.

TABLE 2

ACIDIC AND BASIC GROUPS IN α-KERATOSE FROM PERACETIC ACID-OXIDIZED WOOL

Calculated from analytical results of Corfield, Robson, and Skinner (1958)

Acidic Group	Content (μ-equiv/g)	Basic Group	Content (μ-equiv/g)
Aspartic acid	714	Arginine	594
Glutamic acid	1245	Histidine	47
Cysteic acid	425, 606*	Lysine	263
Amide groups	1171	Total basic groups	904
Total acidic groups	1394†	Excess acidic groups	490†

* Value of O'Donnell and Thompson (1959) for α-keratose from performic acid-oxidized wool.

† This value will vary according to the amide N value (see text).

In conclusion it should be pointed out that with continuous washing in distilled water a protein approaches an isoionic condition. If the protein contains a large excess of anionic side-chain groups which differ greatly in their p*K* values, then in washing free from acid those with the higher p*K* values will remain in the unionized condition as acid is removed by water and those with the lower p*K* values will ionize (or remain ionized) in amount equal to the ionized cationic groups in the protein. The data presented here are in complete accord with such a system.

V. ACKNOWLEDGMENTS

The authors are indebted to Dr. J. A. Maclaren for helpful discussions and to Mr. W. B. Hall for the statistical analysis.

VI. References

Alexander, P., Carter, D., Earland, C., and Ford, O. E. (1951).—*Biochem. J.* **48**: 629.

Alexander, P., Fox, M., and Hudson, R. F. (1951).—*Biochem. J.* **49**: 129.

Alexander, P., and Hudson, R. F. (1954).—"Wool: Its Chemistry and Physics." (Chapman & Hall Ltd.: London.)

Andrews, S., and Schmidt, C. L. A. (1927).—*J. Biol. Chem.* **73**: 651.

Bradbury, J. H. (1958).—*Biochem. J.* **68**: 482.

Cavallino, D., De Marco, C., Mondovi, B., and Stirpe, F. (1958).—*R. C. Accad. Lincei* **18**: 552. (*Chem. Abstr.* **52**: 17101 (1958).)

Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943).—*Biochem. J.* **37**: 354.

Corfield, M. C., and Robson, A. (1955).—*Biochem. J.* **59**: 62.

Corfield, M. C., Robson, A., and Skinner, B. (1958).—*Biochem. J.* **68**: 348.

Earland, C., and Knight, C. S. (1955).—*Biochim. Biophys. Acta* **17**: 457.

Hale, D. K., and Reichenberg, D. (1949).—*Disc. Faraday Soc.* **7**: 79.

Jacobsen, C. F., and Léonis, J. (1951).—*C.R. Lab. Carlsberg* **27**: 333.

Kunin, R., and Barry, R. E. (1949).—*Ind. Engng. Chem.* **41**: 1269.

Lavine, T. F. (1936).—*J. Biol. Chem.* **113**: 583.

Leach, S. J., and Parkhill, E. M. J. (1956).—Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. C. p.C–92.

Lemin, D. R., and Vickerstaff, T. (1946).—"Symposium on Fibrous Proteins." p. 129. (Soc. Dy. Col.: Bradford.)

O'Donnell, I. J., and Thompson, E. O. P. (1959).—*Aust. J. Biol. Sci.* **12**: 294.

Sanger, F. (1949*a*).—*Biochem. J.* **44**: 126.

Sanger, F. (1949*b*).—*Biochem. J.* **45**: 563.

Simmonds, D. H. (1956).—Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. C. p.C–65.

Steinhardt, J., and Fugitt, C. H. (1942).—*Bur. Stand. J. Res., Wash.* **29**: 315.

Thompson, E. O. P. (1959).—*Aust. J. Biol. Sci.* **12**: 303.

Thompson, E. O. P., and O'Donnell, I. J. (1959).—*Aust. J. Biol. Sci.* **12**: 282.

Vogel, A. I. (1945).—"Qualitative Chemical Analysis." 3rd Ed. (Longmans, Green and Co.: London.)

Weston, G. J. (1955).—*Biochim. Biophys. Acta* **17**: 462.

GENETIC SAMPLING IN A RANDOM MATING POPULATION OF CONSTANT SIZE AND SEX RATIO

By B. D. H. Latter*

[*Manuscript received March 23, 1959*]

Summary

An expression is derived for the effective breeding size of a random mating population of constant size and sex ratio, which takes into account reproductive inequalities among the breeding individuals. Consideration is given to the problem of estimating the possible genetic drift in the mean of a given quantitative character in an experimental control population.

I. Introduction

The practice of maintaining a control population of animals, to provide a reference point over a period of generations, is becoming increasingly popular in selection programmes wherever the necessary facilities are available. Whether one is interested to assess short-term fluctuations in the effects of environmental factors on a particular quantitative trait, or to detect and measure long-term trends due to improvements in management, the problem is to hold as constant as possible the genetic constitution of the base population. Gene frequencies in the population will, however, be expected to change from generation to generation, due to the sampling of genes involved in the choice of parents, and to the effects of natural selection. The ways in which the effects of these two agencies can be minimized have been discussed with particular reference to poultry flocks by Gowe, Robertson, and Latter (1959).

If one is able to tag the offspring of each mating within the population and control the contributions of the respective families to the next set of selected parents, the efficiency of such a procedure in reducing the effects of genetic sampling can readily be calculated. It is in fact possible to achieve substantial gains in efficiency in this way, by ensuring that each parent contributes equally to the succeeding set of parents. If, however, the parents are randomly chosen each generation from the survivors to breeding age, changes in gene frequency due to genetic sampling will obviously be a function of differences in reproductive capacity among the breeding individuals. The purpose of this paper is to discuss the genetic sampling process in the latter situation, and to derive a general expression for the effective breeding size of the population. The expression has previously been given without proof by Gowe, Robertson, and Latter (1959).

II. General Approach

The general approach to problems of drift in gene frequency may be illustrated by the derivation (due to Dr. A. Robertson) which follows. Suppose we have N parents from which we sample $n_1, n_2, \ldots, n_N$ gametes respectively, and we consider

---

* Division of Plant Industry, C.S.I.R.O., Canberra.

only sampling between individuals in its effect on the change in gene frequency. If q_i is the gene frequency at a particular locus in the ith parent (the possible values of q_i being 0, $\frac{1}{2}$, or 1), then the change in gene frequency due to sampling between individuals is

$$\delta q' = \sum_i q_i\left(\frac{n_i}{\Sigma n} - \frac{1}{N}\right),$$

and the expected drift variance is therefore

$$E(\delta q')^2 = \sigma_q^2\, E\left[\sum_i\left(\frac{n_i}{\Sigma n} - \frac{1}{N}\right)^2\right]$$

$$= \frac{q(1-q)}{2}\left[\frac{N\sigma_n^2}{(\Sigma n)^2}\right],$$

where q and σ_q^2 are the mean and variance of the q_i, and σ_n^2 the variance of the n_i. In addition, the sampling of gametes within individuals contributes to the drift variance whenever an individual is heterozygous at the locus concerned, giving a total drift variance of

$$E(\delta q)^2 = \frac{q(1-q)}{2}\left[\frac{1}{\Sigma n} + \frac{N\sigma_n^2}{(\Sigma n)^2}\right]. \qquad \ldots\ldots\ldots\ldots\ldots(1)$$

III. Derivation

The population structure with which we shall be concerned is one in which M males and F females are chosen as parents randomly from the surviving animals each generation, and are mated at random to produce the members of the next generation. The overall change in gene frequency from one generation to the next involves genetic sampling along the four paths from male parents to succeeding male parents, from male parents to succeeding female parents, and so on, and we must take account of the fact that the changes in gene frequency along the separate paths are not necessarily uncorrelated.

Consider M male parents contributing x_i males to the next generation of male parents and y_i females to the next generation of female parents ($i = 1, 2, \ldots, M$). Suppose x_i has mean μ_1 and variance σ_1^2, and y_i has mean μ_2 and variance σ_2^2. Denoting by δq_1 the change in gene frequency along the path male → male, and by δq_2 the change along the path male → female, we have from (1)

$$E(\delta q_1)^2 = \frac{q(1-q)}{2M\mu_1}\left[1 + \frac{\sigma_1^2}{\mu_1}\right],$$

and $\qquad \ldots\ldots\ldots\ldots\ldots\ldots(2)$

$$E(\delta q_2)^2 = \frac{q(1-q)}{2M\mu_2}\left[1 + \frac{\sigma_2^2}{\mu_2}\right].$$

Since the overall change in gene frequency due to sampling from males is $\delta q_m = \frac{1}{2}(\delta q_1 + \delta q_2)$, the expected value of $(\delta q_m)^2$ involves the covariance of δq_1

and δq_2. It is obvious that only sampling between individuals is involved in this covariance, and we have

$$\delta q_1' = \sum_i q_i\left(\frac{x_i}{M\mu_1} - \frac{1}{M}\right),$$

$$\delta q_2' = \sum_i q_i\left(\frac{y_i}{M\mu_2} - \frac{1}{M}\right),$$

so that the expected value of $(\delta q_1)(\delta q_2)$ is

$$E(\delta q_1)(\delta q_2) = \frac{q(1-q)}{2} E\left[\sum_i\left(\frac{x_i}{M\mu_1} - \frac{1}{M}\right)\left(\frac{y_i}{M\mu_2} - \frac{1}{M}\right)\right]$$

$$= \frac{q(1-q)}{2M} \cdot \frac{\operatorname{cov}(x_i, y_i)}{\mu_1\mu_2}. \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

If complete pedigrees are available, the required covariance term can be calculated directly, but in order to obtain a general expression for use in the absence of pedigree records, we are lead to postulate the following sequence in the selection of parents in any generation. Suppose each male contributes m_i individuals regardless of sex to a group of progeny which is to be sampled at random (separately within each sex) to provide the succeeding M male and F female parents. Let the mean and variance of m be μ_m, σ_m^2. The m_i offspring of the ith male will then be distributed to the two sexes according to a binomial distribution with parameters m_i and $\frac{1}{2}$ denoted $B\ (m_i, \frac{1}{2})$. If the actual split is n_i males and m_i-n_i females, the probabilities that x_i, y_i individuals are included in the sample following random choice of M males and F females are given by $B\ (n_i, \mu_1/\frac{1}{2}\mu_m)$ and $B\ (m_i-n_i, \mu_2/\frac{1}{2}\mu_m)$.

The required covariance is then

$$\operatorname{cov}(x_i, y_i) = [(\mu_1\mu_2)/\tfrac{1}{4}\mu_m^2] \operatorname{cov}(n_i, m_i-n_i)$$

$$= \mu_1\mu_2\left(\frac{\sigma_m^2-\mu_m}{\mu_m^2}\right).$$

Before combining expressions (2) and (3), we must derive a relationship between σ_m^2 and σ_1^2, σ_2^2. Within the framework of the above assumptions, it turns out that

$$\sigma_1^2 = \mu_1\left[1 + \mu_1\left(\frac{\sigma_m^2-\mu_m}{\mu_m^2}\right)\right],$$

and

$$\sigma_2^2 = \mu_2\left[1 + \mu_2\left(\frac{\sigma_m^2-\mu_m}{\mu_m^2}\right)\right],$$

so that we can write

$$E(\delta q_m)^2 = \frac{q(1-q)}{4}\left[\frac{1}{M\mu_1} + \frac{1}{M\mu_2} + \frac{2R_m}{M}\right],$$

where $R_m = (\sigma_m^2 - \mu_m)/\mu_m^2$. In a population of constant size and sex ratio, $\mu_1 = 1$ and $\mu_2 = F/M$, and the expression becomes

$$E(\delta q_m)^2 = \frac{q(1-q)}{4}\left[\frac{1}{M} + \frac{1}{F} + \frac{2R_m}{M}\right]. \quad \ldots\ldots\ldots\ldots(4)$$

A similar expression can be derived for the drift variance due to sampling along the two paths from female → male and female → female, and these gene frequency changes are obviously independent of the two already considered. We have

$$E(\delta q_f)^2 = \frac{q(1-q)}{4}\left[\frac{1}{M} + \frac{1}{F} + \frac{2R_f}{F}\right], \quad \ldots\ldots\ldots\ldots(5)$$

where $R_f = (\sigma_f^2 - \mu_f)/\mu_f^2$, μ_f and σ_f^2 being defined in the same manner as μ_m, σ_m^2. Combining (**4**) and (**5**), the average change in gene frequency from one generation to the next has variance

$$E[\tfrac{1}{2}\delta q_m + \tfrac{1}{2}\delta q_f]^2 = q(1-q)\left[\frac{1}{8M}(1+R_m) + \frac{1}{8F}(1+R_f)\right]. \quad \ldots(6)$$

The derivation of expression (**6**) has been based on two assumptions in addition to the stipulation that chosen parents be randomly mated each generation; they are:

(1) That the group of progeny to which the parameters μ_m, σ_m^2 and the corresponding μ_f, σ_f^2 refer shall be sampled at random separately within each sex to provide M male and F female breeding individuals each generation; and

(2) That mortality in the population, prior to the point of time to which the abovementioned parameters refer, shall have been independent of sex.

With complete equality among the male parents and among the female parents in reproductive capacity, and random mortality among the offspring, each parent has an equal chance of contributing to the next set of selected parents, and the anticipated distribution of progeny number per parent is one in which the mean and variance are equal (Poisson), i.e. R_m and R_f are zero. Expression (**6**) then reduces to the familiar formula

$$E(\delta q)^2 = q(1-q)\left[\frac{1}{8M} + \frac{1}{8F}\right].$$

IV. DISCUSSION

An expression has been given by Crow and Morton (1955) for the expected drift variance in gene frequency in a population with unequal numbers in the two sexes which at first sight seemed to provide the solution to the problem we have posed. In the above notation, their equation can be written

$$E(\delta q_m)^2 = \frac{q(1-q)}{2M(\mu_1+\mu_2)}\left[1 + \frac{\mathrm{var}(x_i+y_i)}{\mu_1+\mu_2}\right],$$

which is a direct application of expression (1) above. In the framework we have specified above, this reduces to

$$E(\delta q_m)^2 = \frac{q(1-q)}{4}\left[\frac{4}{M+F}+\frac{2R_m}{M}\right],$$

which is only equivalent to (4) if the numbers of male and female parents in the population are equal. The difference stems from the fact that Crow and Morton have taken the average gene frequency following sampling as a weighted average of the gene frequencies in males and females, whereas the average relevant to our problem is unweighted. Presumably their expression was not intended to apply to a population in which the sex ratio is held constant from generation to generation.

The problem of estimating the possible genetic drift in the mean of a given quantitative character in an experimental control population can best be considered in terms of the "effective" size of the population (N_e), defined such that

$$E(\delta q)^2 = \frac{q(1-q)}{2N_e}.$$

One can also speak of the effective number of male parents (M_e) and the effective number of female parents (F_e), where

$$\frac{1}{N_e} = \frac{1}{4M_e}+\frac{1}{4F_e}.$$

The change in genetic mean performance from one generation to the next due to genetic sampling has variance roughly equal to σ_g^2/N_e where σ_g^2 is the additive genetic variance in the character concerned, so that an estimate of effective population size can be used to provide some measure of the genetic changes in the population mean which are likely over any given period of generations. Such a measure will of course take no account of the effects of natural selection over the period concerned, and is likely to be most useful in the case of populations with no immediate history of artificial selection.

An instance of this approach has been given by Gowe and Johnson (1959). The data which these authors had available were the mean number of eggs per hen laid during the period from which replacements were kept, and the corresponding variance; and the mean hatchability per hen over the same period, with the corresponding variance. Making the assumption that production and hatchability were uncorrelated, it was possible to estimate the mean and variance of number of chicks per hen, and to make use of these statistics to estimate the effective number of females in the control flock concerned. Use of the expression given in this paper was conditional upon the assumptions that mortality from hatching to breeding age was random within each sex, and that embryonic mortality was independent of sex.

V. ACKNOWLEDGMENTS

My thanks are due to Dr. A. Robertson, Institute of Animal Genetics, Edinburgh, and to Dr. R. S. Gowe, Central Experimental Farm, Ottawa, for guidance and stimulating discussion. The award of a Walter and Eliza Hall Agricultural Research Fellowship from the University of Sydney is gratefully acknowledged.

VI. References

Crow, J. F., and Morton, N. E. (1955).—Measurement of gene frequency drift in small populations. *Evolution* **9**: 202–14.

Gowe, R. S., and Johnson, A. S. (1959).—Environment and poultry breeding problems. IV. The value of a random-bred control strain in a selection study. *Poult. Sci.* **38**: 443–62.

Gowe, R. S., Robertson, A., and Latter, B. D. H. (1959).—Environment and poultry breeding problems. V. The design of poultry control strains. *Poult. Sci.* **38**: 462–71.

SELECTION FOR AN INVARIANT CHARACTER, VIBRISSA NUMBER, IN THE HOUSE MOUSE

By R. B. Dun* and A. S. Fraser†

[*Manuscript received December* 22, 1958]

Summary

The number of secondary vibrissae in the mouse is normally 19. The tabby gene reduces this to about half and causes a marked variability of vibrissa number. Selection on tabby mice for increase and decrease in vibrissa number has been successful. These selection lines were maintained in segregation for + and *Ta* alleles, and this selection, practised solely on tabby mice, has resulted in both an increase and decrease in vibrissa number in normal mice.

I. Introduction

It has been generally accepted that an absence of phenotypic variation has its basis in an absence of genetic variation, i.e. phenotypic invariability implies genetic fixation. Goldschmidt (1935), Landauer (1958) and others, in their researches on the induction of phenocopies, concluded that considerable genetic variation occurs which is only manifested phenotypically in phenocopies, i.e. this genetic variation is only manifested after some environmental stress has markedly affected development. Waddington (1956) extended their observations showing that genetic variation which was only manifested in phenocopies could be selected until it was manifested in the absence of the specific environmental stress.

A further extension of this theme is examined in this paper. In general, mutant genes have a much greater phenotypic variability than their normal alleles. We have taken as a basis for our researches the number of secondary vibrissae in the house mouse, which is remarkably constant in normal mice, but highly variable in the presence of certain mutant genes. Consequently selection for high or low vibrissa number, which is difficult or impossible in normal mice, is quite feasible in mice carrying one of these mutant genes. Such selection if continued long enough can result in differences of vibrissa number in normal mice. We are, in other words, employing the stress of a mutant gene instead of an environmental stress as Waddington did.

II. Material and Methods

The character used in this selection experiment was vibrissa number. This is a composite score formed by addition of the five minor groups of vibrissae found on the head and fore limbs. Figure 1 shows the distribution of these vibrissae. The

---

*New South Wales Department of Agriculture, Agricultural Experiment Station, Trangie, N.S.W.

†Animal Genetics Section, C.S.I.R.O., University of Sydney.

basic arrangement is 2 supra-orbitals, 1 postorbital, 2 postorals, 3 inter-ramals, and 3 ulnar–carpals, making a total of 19 vibrissae.

Dun (1958) made an extensive survey of the variation of vibrissa number in 3000 normal mice. These were drawn from several inbred lines and random-bred stocks. Variation from the basic number of 19 was rare. The inter-ramal group was the major source of variability having occasionally two rather than the normal three vibrissae. Several discrete variations were found associated with the supra-orbital group. It is important to note that the postorbital vibrissa was present in all the mice examined, and neither more nor less than one vibrissa was found at each postorbital position. Over all the five groups approximately one abnormality per 500 groups was noted. It can be stated that the number of secondary vibrissae is extremely stable, there being little variation in most groups, and no variation of the postorbital vibrissa.

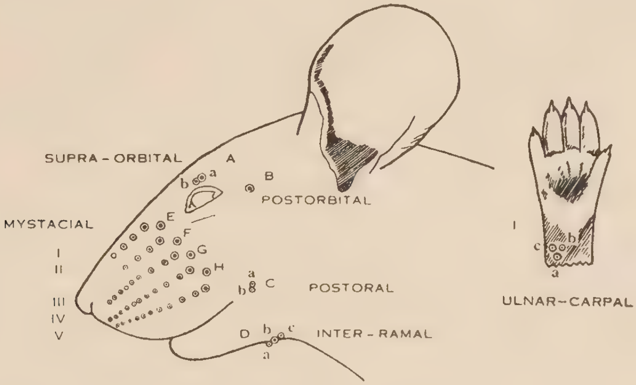


Fig. 1.—Head and distal part of the right fore limb of the mouse showing distribution of vibrissae.

Subsequent to Dun's survey a number of additional mice were scored for vibrissa number. Of 546 Aw101 mice only three showed any variation of vibrissa number, having two instead of three inter-ramal vibrissae. Of 412 CBA mice only one mouse had two instead of three inter-ramals; all other vibrissae were normal. Some 2180 mice from the cross of the DBA line with the CBA and Aw101 lines were scored. In none of these was the postorbital missing. Totalling all the mice examined, over 12,000 postorbital groups have been scored without any variation of the basic number being detected. Clearly, it is safe to state that the number of postorbital vibrissae is an invariant character.

Whereas Waddington (1953) exposed genetic variation to selection by subjecting the animals to an environmental stress during development, we have used the sex-linked semidominant mutant gene tabby (*Ta*) (Falconer 1953) to expose genetic variation in vibrissa number.

The ranges of vibrissa number in normal mice and in *Ta* + (♀) and *Ta*· (♂) mice are given in Figure 2, which shows the wide range of vibrissa numbers occurring in tabby mice. It was this variation which was utilized in our selection experiment.

These data summarize the widest possible ranges of vibrissa number in normal mice, including all stocks and lines, and are shown separately for the various inbred lines and the TA stock in Table 1. The TA stock is a crossbred stock in which the tabby gene is kept segregating. In these TA stocks the + segregants show less phenotypic variation than in the whole survey of non-TA stocks. It is clear that the variability of vibrissa number is marked in tabby mice and almost non-existent in + mice. It seems reasonable to claim that any genetic variation determining vibrissa number in tabby mice also occurs in + mice. If it can be shown that + mice respond to selection based on the variation of tabby mice this point will be proved. It is a fair inference that, as the TA stocks were built up from backcrosses and intercrosses of the other stocks listed in Table 1, background variation present in + animals of the TA stocks came from the other + stocks and is present in them.

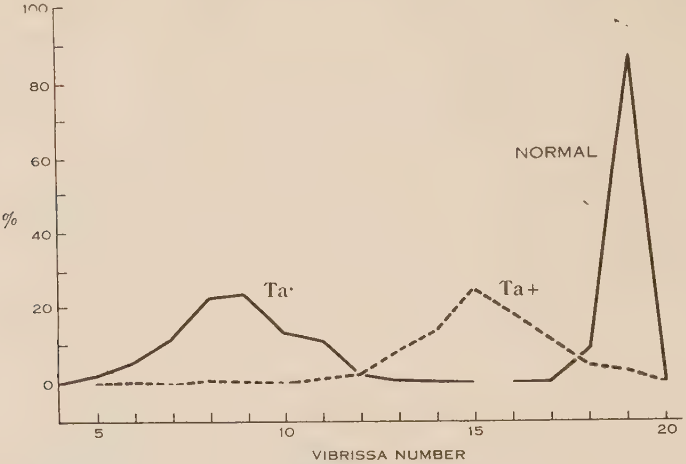


Fig. 2.—Range of vibrissa number in normal mice compared with the range in *Ta*+ females and *Ta*· males.

TABLE 1

FREQUENCIES OF NUMBERS OF VIBRISSAE IN INBRED LINES, AND IN THE NORMAL MICE OF THE TA STOCK

Stock	Supra-orbitals			Post-orbitals		Postorals				Inter-ramals					Ulnar–Carpals			
	2	1	0	1	0	3	2	1	0	4	3	2	1	0	3	2	1	0
Aw101	459	1	–	460	–	–	459	–	–	–	230	–	–	–	460	–	–	–
CBA	496	–	–	496	–	–	496	–	–	–	245	3	–	–	496	–	–	–
C_3H	403	–	–	408	–	–	407	–	–	2	194	8	–	–	407	1	–	–
C_{57}	324	–	–	324	–	–	324	–	–	2	137	22	1	–	324	–	–	–
DBA	308	–	–	308	–	1	307	–	–	–	98	56	–	–	308	–	–	–
A	231	1	–	232	–	–	231	–	–	6	47	62	1	–	223	9	–	–
TA(+)	238	–	–	238	–	–	238	–	–	–	108	10	1	–	90	–	–	–

(*a*) *The Selection Stock*

A basic selection stock, the ST stock, was formed by crossing mice from the following lines:

(1) HF (2) MH (3) TA	Tabby stocks	(4) CBA (5) Aw101	Inbred lines

HF and MH were stocks developed for histological work, which were established from the original tabby stock (the TA stock). The latter was obtained from the Institute of Animal Genetics, Edinburgh. Both the CBA and Aw101 stocks were frequently used in the formation of the HF and MH stocks.

Table 2

PERCENTAGE REPRESENTATION OF FIVE STRAINS OF MICE IN THE FOUNDATION STOCK ST AND IN THE SELECTION LINES HST AND LST AT GENERATIONS 1 AND 4

Selection Lines	Strains of Mice				
	HF	MH	TA	Aw101	CBA
ST	28·6	21·4	19·0	16·7	14·3
HST, generation 1	8·9	42·9	1·8	44·6	1·8
LST, generation 1	16·1	19·6	41·1	3·6	19·6
HST, generation 4	4·2	47·6	0	47·6	0·6
LST, generation 4	19·4	19·4	36·6	0	24·6

In the initial matings to form the ST stock, 21 *Ta*+ females from the three tabby stocks were mated with normal males: 8 from the HF, MH, and TA stocks and 13 from the Aw101 and CBA stocks. These matings produced *Ta*+ females which were selected for high and low vibrissa number to establish the two selection lines: HST (high selection) and LST (low selection). These *Ta*+ females were mated to + males which had been selected on the performance of their *Ta*+ sisters. The result was a mixture of mouse strains in the initial phase of the experiment. Table 2 shows the percentage representation of each stock in the pedigrees of the foundation ST stock, and in the HST and LST selection lines at generations 1 and 4.

The Aw101 and MH stocks were predominant in the foundation of the HST selection line, whereas the TA and CBA stocks were predominant in the foundation of the LST selection line. This asymmetry in genetic constitution of the two lines means that no especial significance can be attributed to any asymmetry of response to selection.

Sib-matings were avoided throughout the experiment. With a population size of 10 males and 30 females one would expect a loss of 1·7 per cent. of the initial heterozygosity per generation. The actual loss was nearly double this: the doubling results from the use of sib-testing in the selection of normal males which reduces the effective number of males.

(*b*) *The Method of Selection*

Mice were initially selected by a combination of mass selection of *Ta*+ females and sib-testing of + males. It was impossible to select + males on their own phenotype, since they showed no, or very little, variation—certainly insufficient to allow useful selection.

There were 12 pairs of selected mice mated in generation 1, 18 in generation 2. At this stage dissatisfaction was felt with the results which were being obtained. Accordingly females were subsequently allowed to have two litters: the first to provide animals for selection, the second to provide additional data. In addition the mating scheme shown below was adopted:

	Females		Males	
(1)	*Ta*+	×	+·	Parents
	Ta+, ++		*Ta*·, +·	Progeny
(2)	++	×	*Ta*·	Parents
	Ta+		+·	Progeny
(3)	*Ta*+	×	+·	Parents

and so on. This mating scheme is self-continuing and has the marked advantage of producing + mice every generation.

Table 3

HERITABILITIES AND STANDARD ERRORS OF VIBRISSA NUMBER

Genotype Scored	Sires h^2(S)	Component Degrees of Freedom	Dams h^2(D)	Component Degrees of Freedom	$h^2\left(\frac{S+D}{2}\right)$
Tabby females (*Ta*+)	0·05±0·11	11	0·78±0·33	22	0·42
Tabby males (*Ta*·)	0·44±0·24	11	0·70±0·33	22	0·57

Initially these + mice showed no useful degree of variation. At later generations, when + mice occurred with aberrant numbers of vibrissae, such mice were only accepted as parents where this was indicated from the vibrissa number of their tabby sibs, i.e. in the whole experiment no selection has been practised directly on phenotypes of + mice.

It can be seen from Table 1 that there is some variation of the frequency of inter-ramals in normal mice. Consequently a second selection experiment was initiated in which selection pressure was set to cause both increase and decrease of the frequency of inter-ramals (the two selection lines are termed HIR and LIR respectively). The aim of this experiment was to demonstrate that selection on inter-ramals in normal mice could not produce the same changes of total number of vibrissae that were found in the tabby selection experiment.

(c) *Heritability of Vibrissae Number*

The heritability of vibrissae number in normal mice can be taken as effectively zero. In *Ta*+ and *Ta*· mice, the heritability was first estimated by analysis of the components of variance for full and half-sibs. The standard errors of the estimates were calculated using the method of intraclass correlations (Fisher 1946). Results are set out in Table 3.

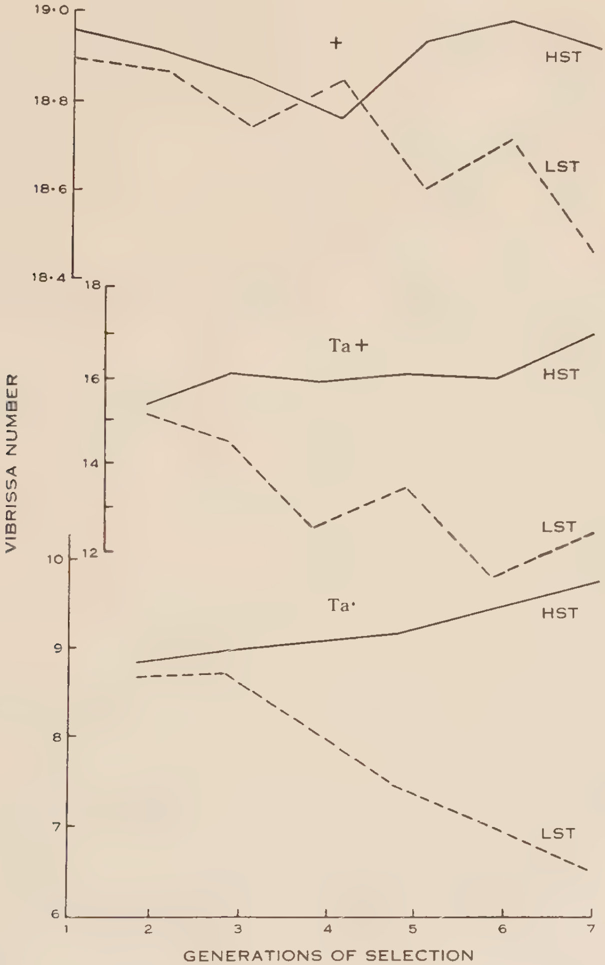


Fig. 3.—Mean vibrissa number at the three levels of expression plotted against generation for the tabby selection experiment: each level of expression is shown plotted against a different scale to emphasize the differences between HST and LST.

TABLE 4

CHANGES IN THE MEAN AND VARIANCE OF VIBRISSA NUMBER IN *Ta*+ AND *Ta*· MICE DURING SEVEN GENERATIONS OF SELECTION

Parameter	Selection Line	Basic Selection Stock (ST)	Generation						
			1	2	3	4	5	6	7
Ta+ mice									
Number of mice	HST	76	50	128	39	99	45	125	50
	LST		40	99	48	109	38	112	46
Mean	HST	8·90	9·92	15·27	16·00	15·79	16·00	16·02	17·02
	LST		8·90	15·06	14·46	12·61	13·63	11·42	12·48
Variance	HST	1·372	2·786	3·759	2·263	3·680	4·318	4·040	2·225
	LST		2·349	3·980	4·083	6·080	3·374	5·670	5·233
Coefficient of variation (%)	HST	13·2	16·8	12·7	9·4	12·2	13·0	12·5	8·8
	LST		17·2	13·3	14·0	19·6	13·5	20·8	18·3
Ta· mice									
Number of mice	HST			132	34		41		37
	LST			124	29		34		34
Mean	HST			8·81	9·00		9·29		9·81
	LST			8·62	8·72		7·35		6·59
Variance	HST			2·770	4·570		3·676		2·992
	LST			2·550	1·890		1·447		0·915
Coefficient of variation (%)	HST			19·0	23·8		20·7		17·6
	LST			18·6	15·8		16·3		14·5

Using the parent–offspring regression, the heritability of total vibrissae number was $0 \cdot 36 \pm 0 \cdot 24$. Unfortunately the errors of these estimates were high, but the agreement between estimates from the two methods suggests that the heritability lies in the range 0·3–0·5. It is sufficiently high to suggest that mass selection would be effective.

III. RESULTS

(*a*) *Effects of Selection*

The tabby selection experiment has three levels of expression: (1) the + level; (2) the *Ta*+ level; and (3) the *Ta*· level. The mean values at each generation for all three levels are shown in Figure 6, which since only a single scale is used allows comparison of the effects of selection between the three levels. The data are also plotted in Figure 3 on scales which are different for the three levels in order to show more clearly the rather small changes which have occurred at the + level.

Selection has been successful in both directions in both *Ta*+ and *Ta*· mice. At the *Ta*+ level the lines have separated by approximately 3 standard deviations by generation 7. The change in LST has been at twice the rate of the change in HST, the regression coefficients being $-0 \cdot 60 \pm 1 \cdot 32$ and $0 \cdot 25 \pm 0 \cdot 34$ respectively. The response to selection at the *Ta*· level closely parallels that of the *Ta*+ level.

It is interesting to note that there is a tendency in both the HST and LST lines for vibrissa number to increase in generations 3, 5, and 7, while the reverse applies in generations 4 and 6. It is likely that these fluctuations are in some way associated with the mating scheme, and it is probable that a maternal effect is involved. This is being investigated.

The variance of vibrissa number is affected by selection. The means, variances, and coefficients of variation of the two tabby levels are given in Table 4. At the *Ta*+ level there is a tendency for the variance to decrease in the high line and increase in the low line; conversely, at the *Ta*· level there is a tendency for the variance to increase in the high line and decrease in the low line. These changes of variance can be explained by the existence of an upper limit to the phenotypic expression of genetic variation at 19 vibrissae, and a lower barrier at 5–6 vibrissae. At the *Ta*+ level the high selection is moving the distribution against the upper barrier, and at the *Ta*· level the low selection is moving the distribution against the lower barrier. The changes of variance are not sufficiently large to justify this model being accepted unequivocally and further selection has, as one of its aims, the investigation of these changes of variance.

Detailed presentation of the effects of selection on the separate groups of vibrissae are given in Dun (1959*a*). The groups which show the maximum response to selection are those which are affected by tabby such that the number of vibrissae is well removed from either the upper or lower barriers to phenotypic expression. For example, the number of supra-orbital bristles in *Ta*+ mice is close to the upper limit of 4, and selection in a high direction had little success, whereas it was possible to depress the supra-orbital score fairly markedly in the low line. The reverse of this situation is seen with the inter-ramal group. In unselected *Ta*+ mice, the number of inter-ramals is near the lower limit of one vibrissa. Consequently selection downwards has been ineffective compared to selection for an increased number of inter-ramals.

Changes in the expression of the + genotypes are based on male scores which were made every generation, and female scores which were made on all generations except 4 and 6 when no + females were born. The + genotypes of the high- and low-selection lines have become clearly separated on vibrissa number: they differ by almost half a vibrissa. At generation 4, LST was temporarily higher than HST. This was entirely due to changes in the inter-ramal group: the HST having a larger number with 2 instead of 3. Mice with two inter-ramals are found from time to time in unselected stocks and to avoid the possibility that chance variations in this will mask changes in other groups in the early stages of selection, the remaining groups have been shown separately in Figure 4. In this figure the percentages of mice which had unusually high or low vibrissa numbers are shown for each generation, excluding the inter-ramal group.

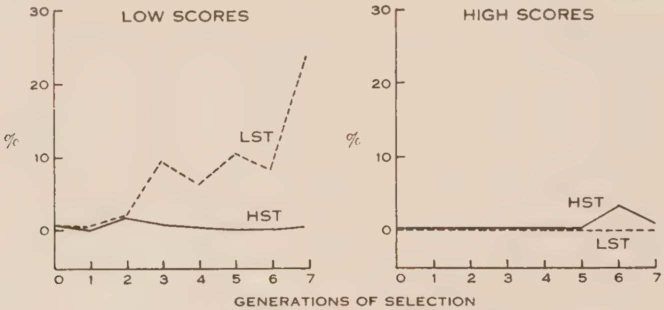


Fig. 4.—Incidence of normal mice having either abnormally low or abnormally high vibrissa number during the course of the selection experiment.

It is apparent that commencing at generation 4 there has been a progressive increase in the number of + mice in the low line which have a lower number of vibrissae than is normally found in unselected stocks. Conversely, commencing at generation 6, the first mice with extra vibrissae occurred in + mice from the high line.

The specific vibrissae involved in these changes are of some interest and each group will now be dealt with in turn. The supra-orbitals showed little change to generation 5, when five normal mice were recorded from the low line with supra-orbital *b* missing. This in itself was unusual as extensive examinations of normal mice had shown that it was a rare occurrence to find one such mouse. Two of these mice also lacked a postorbital vibrissa. This is a convincing demonstration that the normally uniform development of vibrissae had been upset. In generation 6, three mice with an extra supra-orbital vibrissa were found in the high line.

The postorbital group is of particular interest because, as already mentioned, it has been found to consist of one vibrissa in over 12,000 groups examined. The first change at this site occurred in the low line in generation 5 (see above). Two further cases were seen in the low line at generation 6 and one of these also lacked a supra-orbital *b*.

Although occasional mice lacked a postorbital vibrissa (three in the high line, and 10 in the low line), it is not certain that a change in normal variation of this group has yet occurred. Changes in the course of development of the inter-ramals were also less easily established because of the natural occurrence of inter-ramal groups with both 2 and 3 vibrissae. However, several unusual scores were recorded which follow the pattern established by the less variable groups. In generation 3 of the low line, two mice were recorded with only one inter-ramal vibrissa, and in the high-selection line, one mouse in each of generations 6 and 7 had four.

The group in which the selection lines differed most was the ulnar–carpal group. In generation 7 of the low line, no less than 29 groups had two instead of three ulnar–carpals, vibrissa *b* not developing.

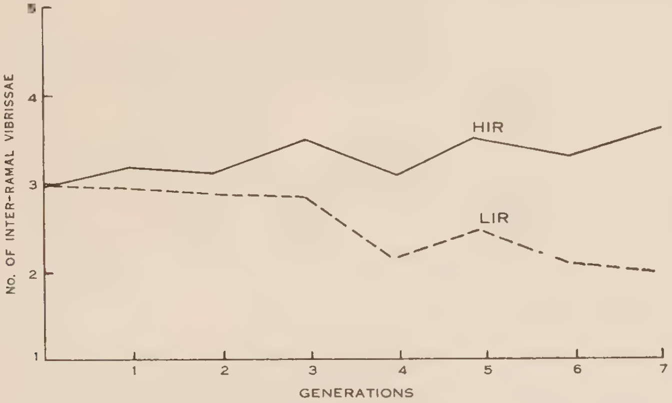


Fig. 5.—Seven generations of selection for and against number of inter-ramal vibrissae.

These data establish our primary thesis: namely, that genetic variation which is either only slightly, or not at all, expressed in + mice is expressed in tabby mice and can be selected at the tabby levels to such extremes that it is manifested in + mice, i.e. in the absence of the tabby gene. However, although variation of vibrissa number is remarkably low, the inter-ramal group does vary, and it can be argued that our selection has been on this group, producing correlated responses on the other groups. To check this point, a selection experiment was initiated based on + mice in which selection was practised for the number of inter-ramals. Figure 5 shows the mean numbers of inter-ramals at the seven generations of selection. By generation 7 the high line had 1·5 more inter-ramal vibrissae than the low line. The point of interest is that there were virtually no associated changes in other groups. The only unusual scores are shown in Table 5.

This shows that occasionally mice have been observed with vibrissae missing at the supra-orbital, postoral, and ulnar–carpal sites. The incidence of missing vibrissae was 1 in 1618 groups for supra-orbitals, 0 for postorbitals, 1 in 809 groups for post-orals, and 1 in 324 groups for ulnar–carpals. These rates are similar to those found in unselected + mice, and it is extremely unlikely that they indicate any change in frequency due to the selection on the inter-ramals.

(*b*) *Correlated Responses in the Tabby Selection Experiment*

Although selection has been applied only on the secondary groups of vibrissae it is of interest to determine whether correlated changes have been produced in the primary group of vibrissae, i.e. the mystacial group. In general, the results show that mystacial vibrissae have, as yet, been little affected by selection. There is very little difference between + mice in the two selection lines.

Table 5

CHANGES IN OTHER GROUPS OF VIBRISSAE FOLLOWING SELECTION FOR HIGH AND LOW INTER-RAMAL NUMBER

+ mice

Selection Line	Groups of Vibrissae			
	Supra-orbital	Postorbital	Postoral	Ulnar–Carpal
High line	One mouse in generation 1 with *b* missing (unilateral)	Invariably present	One mouse in generation 3 with *b* missing (unilateral) One mouse in generation 4 as above	Three mice in generation 1 with *b* missing (unilateral)
Low line	Invariably present	Invariably present	Invariably present	Two mice in generation 4 with *b* missing (unilateral)

Table 6

SCORES FOR INTENSITY OF COAT STRIPING IN *Ta*+ MICE AT GENERATIONS 2 AND 7 OF THE TABBY SELECTION EXPERIMENT

Generation	Group	Number of Mice	Mean Tabby Score	Standard Error
2	HST	37	4·02	0·29
	LST	27	4·78	0·32
7	HST	36	3·42	0·27
	LST	36	5·03	0·32

Although we have considered the effect of the tabby gene on the secondary groups of vibrissae, this gene is usually scored from its effect on the coat. In *Ta*+ mice there is a marked striping of the colour of the coat. In *TaTa* and *Ta*· mice the structure of the coat is markedly changed, mimicking the structure of the coat of crinkled mice. It is of interest to determine whether selection on one aspect of the tabby phenotype (the number of vibrissae) produces correlated effects on another aspect, the coloration of the coat in *Ta*+ mice. A set of photographs of *Ta*+ females were taken and graded visually on a nine-point scale. Coats scored 1 were indistinguishable from normal, while coats scored 9 were indistinguishable from hemizygous tabby males. Mice were scored in generations 2 and 7, and the mean scores are given in Table 6.

It is evident that a change in dominance of the tabby gene is accompanying the alterations of vibrissa number. This is to be expected as Dun (1959*b*) has shown that there is a negative correlation between vibrissa number and tabby score. *Ta*+ females in the high line are tending to a low score, i.e. tabby is becoming recessive. In the low line, selection is changing tabby towards full dominance.

TABLE 7

BODY WEIGHTS AND TAIL LENGTHS OF MICE AT GENERATION 7 OF THE TABBY SELECTION EXPERIMENT

Genotype	Selection Line	Body Weight (g)		Tail Length (cm)
		28 Days	56 Days	
++ (♀)	HST	15·13±0·25	21·31±0·29	9·42±0·05
	LST	15·27±0·30	19·31±0·30	9·26±0·06
Ta+ (♀)	HST	14·14±0·33	21·59±0·29	9·25±0·05
	LST	14·61±0·35	18·84±0·33	9·21±0·05
+· (♂)	HST	15·03±0·28	25·26±0·33	9·52±0·05
	LST	15·70±0·30	22·44±0·32	9·51±0·05
Ta· (♂)	HST	12·77±0·32	22·73±0·40	8·66±0·07
	LST	11·75±0·47	17·50±0·84	8·25±0·08

At generation 7, all mice born in the selection lines were weighed at 28 and 56 days of age. Tail lengths were also measured at 56 days. The results are summarized in Table 7.

It is interesting to note that the *Ta* gene depresses growth more markedly in the low- than in the high-selection line. + males are 11·3 per cent. heavier than *Ta*· males in the HST line whereas they are 28·3 per cent. heavier in the LST line. At 56 days HST mice of all genotypes are significantly heavier and have longer tails than their LST analogues. This difference is due to a greater rate of weight increase in the post-weaning period.

Two measurements of fertility were recorded each generation: total number of mice per litter, and number of days between commencement of mating and the ensuing parturition. The results are shown in Table 8, giving the average difference between HST and LST females. A positive difference indicates higher figures for the high-selection lines.

HST females have consistently produced larger litters than LST females, the difference reaching significance in generations 3, 4, and 5. The number of days to parturition does not show a general trend in favour of either line. In order to examine the possibility that vibrissa number of *Ta*+ mice was related to fertility (as suggested by the larger litter size in HST), correlations were estimated. Only one correlation reaches significance, but there is a general trend for *Ta*+ mice of high vibrissa number to produce larger litters in a shorter time.

These effects on reproductive fitness may be fortuitous, rather than direct effects of selection on vibrissa number. As an illustration, the frequencies of certain colour genes have changed in the selection lines. Since several colour genes have been shown in other researches to have effects on body size and reproductive fitness, it is possible that the difference of these characters between the selection lines are concomitants of the different frequencies of the colour genes. The different frequencies of colour genes may be fortuitous but it is possible that these genes have effects on vibrissa number, and that the differences of their frequencies between the selection lines are concomitants of the selection on vibrissa number. No effect of these genes on vibrissa number could be detected, but the data were not extensive, and do not exclude this possibility.

TABLE 8

DIFFERENCES IN LITTER SIZE AND DAYS BETWEEN MATING AND PARTURITION BETWEEN HST AND LST FEMALES MATED IN EACH GENERATION OF THE SELECTION EXPERIMENT

Difference in:	Generation						
	1	2	3	4	5	6	7
Litter size	+0·79	+0·35	+0·77*	+0·80*	+1·10*	+0·53	+0·87
Standard error	0·62	0·58	0·38	0·40	0·45	0·42	0·53
Time to parturition (days)	−0·57	−3·39	+0·80	+2·13*	−2·27	+1·10	+0·37
Standard error	1·52	3·97	0·80	0·97	1·26	1·30	1·35
Degrees of freedom	24	30	55	57	56	58	53

*Significant at the 5 per cent. level.

IV. DISCUSSION

Our results can be summarized as showing:

(1) A low variance of vibrissa number in unselected + mice. This could be due to (i) a lack of genetic variation, (ii) constancy of the aspects of the environment which affect vibrissa number, or (iii) canalization of the development of vibrissa such that both genetic and environmental variation are ineffective.

(2) A high variance of vibrissa number in *Ta* mice which could be due to (i) an increased sensitivity to environmental variation caused by the *Ta* gene, (ii)

variation of a genetic system specifically acting only in the presence of *Ta*, i.e. specific modifiers, or (iii) variation of a genetic system whose effects are "canalized" and therefore not expressed phenotypically in + mice but which are expressed in *Ta* mice, because the *Ta* gene reduces or removes the canalization. That there is an increased sensitivity to environmental effects has been shown by Hardy and Fraser (unpublished data) who both found marked maternal effects of the genotype of the dam on the vibrissa number of her *Ta* progeny.

(3) A marked effect of selection on the number of vibrissae in *Ta* mice showing that the variation of vibrissa number in such mice is, to some degree, genetically determined. This is confirmation of the estimate of heritability found in a random-mated population, i.e. 30–50 per cent. It can be argued that the *Ta* is a *mutator*, but since the variation occurs only in vibrissa number this is very unlikely.

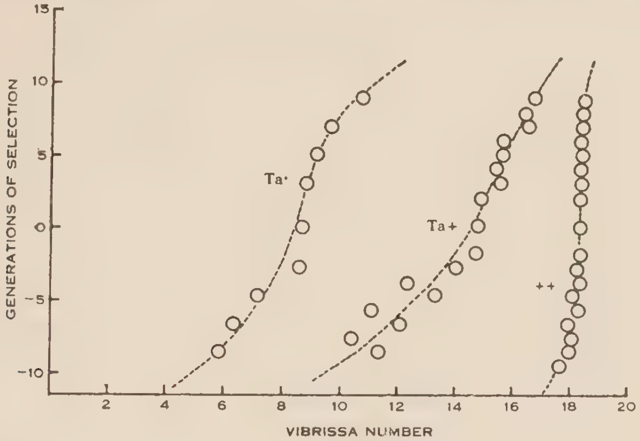


Fig. 6.—Mean vibrissa number is shown plotted against generations of selection for the ++, *Ta*+, and *Ta*· genotypes. This figure should be compared with that given by Dun and Fraser (1958).

(4) That concomitant changes in the other aspects of the tabby phenotype occurred with the changes of vibrissa number. The genetic system affected by selection can be described as one of "dominance modification". There are strong indications that *Ta*+ mice with a high vibrissa number (i.e. approaching normal) produce larger litters in a shorter time. Consequently, the genetic system affects vibrissa number, dominance, and reproductive fitness.

(5) That selection for changes of vibrissa number in *Ta* mice causes concomitant changes in the vibrissa number of + mice, i.e. the genetic system determining variation of vibrissa number in *Ta* mice can, if selected to a sufficient extreme, produce effects on vibrissa number in + mice. This shows that the "dominance modifiers" of *Ta* affect the expression of the + allele, and, consequently, are not specific modifiers of the *Ta* gene. It can be argued that selection on vibrissa number of + mice, in the absence of the *Ta* gene, could affect the dominance of the *Ta* gene. Clearly such an argument has relevance to criticisms of Fisher's theory of dominance, but it is not possible to generalize from this one example of incidental dominance.

Dun and Fraser (1958), in a preliminary note on these results, expressed their conclusions in a diagram, which is shown in a modified form in Figure 6. This is based on the *Ta* locus determining the relationship of the basic genetic system, termed the "vibrissa number system", to the developed number of vibrissae. This relationship is, on this model, a sigmoid function, whose slope and mean are affected by the *Ta* gene. The concept of canalization is, essentially, described by a sigmoid relationship of genotype to phenotype. We concluded that selection shifts a genetic system which determines the mean number of vibrissae.

Rendel (1959), in a similar selection experiment involving the scute gene and the number of scutellar bristles in *Drosophila*, has found an essentially similar system. The standard number of such bristles is 4, and variation is rare. The scute gene causes a reduction to 1–2, and a marked increase of variation around this mean. Selection for increased scutellar number in populations segregating for scute produces changes in the scute flies and this selection, when sufficiently advanced, is correlated with changes in the scutellar number of + sibs. The selection has proceeded further than ours since scute flies with the same number of scutellar bristles as + flies have been produced. As the average number of bristles in scute flies approached "standard" (i.e. 4), there was a marked reduction of variability. Conversely, a marked increase of variability occurred in + flies as their average scutellar number exceeded 4.

Rendel concludes that there is a canalization of scutellar number at 4, i.e. the relationship of phenotype to genotype is sigmoid with a marked inflexion zone at 4 bristles. The results are very similar to ours but Rendel does not consider that the scute gene affects the canalization. On the basis of the model which he proposes, the scute gene is postulated to affect the degree of expression of the "scutellar number" genes directly; not by modifying the sigmoid function relating the basic genetic system to the phenotype. His model is illustrated in Figure 7.

Neither sets of data contain any features which allow discrimination between the two models, i.e. it is equally valid to consider that the tabby and scute genes act directly on the basic *vibrissa number* and *scutellar number* genetic systems, or that they act on the sigmoid relationship of genotype to phenotype. Accurate analyses of the distributions of the characters at various mean numbers of vibrissae or bristles could distinguish between the alternatives for specific types of canalization, but it is probably more useful to compare the developmental tracks affected by the basic genetic systems and the major mutants. The complexity of the systems with which we are dealing is such that both methods should be followed, since neither is likely to be completely conclusive. An extremely interesting feature of Rendel's data is pertinent to this problem. This is the occurrence of scute flies with 4 bristles in which one of the bristle sites was empty. Such flies indicate that the "canalization zone" of 4 bristles is not equally stable across its range since such flies are only observed at the low end of the 4 zone. + flies segregating from the selection experiment do not show any such abnormalities indicating that the lower stabilization (canalization) is not a feature of the middle and high end of the 4 zone, or is manifested in ways which were not scored. It would be of interest to have measurements of the size of bristles in populations located at the edges of the 4 zone, since these might indicate the degree of stability at the edges.

The genetic system determining the number of vibrissae differs markedly in expression between the three genotypes. At the + level the vibrissa number genotype produces very little variation; in the "heritability" terminology, the mode of action of this genotype is almost completely epistatic. At the *Ta* levels, two differences are apparent: firstly, that there is a decrease in the average effect of this genotype, and, secondly, a shift in the mode of action from being almost completely epistatic to its being predominantly additive.

We can describe the *Ta* gene as an "epistasis modifier". We then have a "dominance modifier" system effecting the tabby locus, which is itself an epistasis modifier of the dominance modifier system. Clearly such terminology is becoming too complicated and involved to describe situations like this.

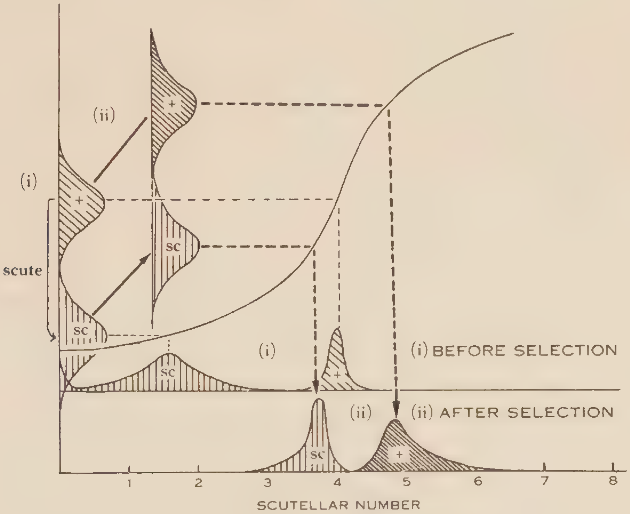


Fig. 7.—Schematic representation of the results and interpretation of Rendel's (1959) selection experiment. Scute is postulated not to affect the sigmoid relationship of genotype/phenotype as has been proposed for the tabby gene, but rather to directly reduce the potency of the basic "scutellar number" genes. The genetic distributions are shown vertically; the phenotypic distributions horizontally.

Consider the genetics of a population which would be produced by crossing our high and low lines. In such a population segregation would occur at the tabby locus, and variation of the vibrissa number system would be marked. The range of vibrissa number would be from 4–5 to 21–22, and in the absence of any secondary effects of the tabby gene it would be difficult to identify the existence of segregation at that locus. Estimates of the "additive" and "epistatic" variance, if based on average values over the whole distribution, would, firstly, give incorrect values of these parameters, and, secondly, give no measure of the complexity of the system. If, however, these parameters were estimated on an assortatively mated population and heritability etc. were determined at several ranges of the phenotype then the complexity of the system would become apparent, and valid predictions could be made of the effects of selection.

An important feature of these experiments is the demonstration of genetic variability in the absence of phenotypic variability. Waddington (1953, 1956), Bateman (1956), Rendel (1959), and our own experiment have all demonstrated this, and it is pertinent to question the assumption that lack of phenotypic variability generation after generation is a demonstration of homozygosis. Although the number of demonstrations of such genetic variability is small, it may be much more widespread than is at present considered possible. It is therefore necessary to consider why such heterozygosity exists. Some results of Fraser (1959), although preliminary, are of interest. It was shown in Monte Carlo simulations of the effects of selection against variability that such selection would form an epistatic system determining a sigmoid relationship of genotype to phenotype, if sufficient genetic variability existed. Even in the absence of genes capable of giving rise to such a system, selection did not produce any marked degree of homozygosity except in small populations.

Although these results require considerable amplification, it is possible to argue from them that the heterozygosity demonstrated in the selection experiments of Waddington, Bateman, Rendel, and Dun and Fraser is a relic. Selection could have acted first on a polygenic system so that this was centred around the desired phenotype. Selection against variability, if the number of loci involved is large, would be ineffective unless directed at other genetic systems capable of determining a sigmoid genotype to phenotype relationship, i.e. canalization. On this hypothesis heterozygosity is a consequence of the inability of optimizing selection to produce homozygosis when acting on a polygenic system rather than a direct determinant of decreased phenotypic variability as proposed by Lerner (1954). Since optimizing selection would favour complex epistatic interactions, the final result would be a genetically variable system with a marked degree of epistasis, in which inbreeding could be expected to result in extreme deviants. Similarly many of the phenomena taken by Lerner (1954) to indicate that homeostasis has a heterozygous basis could be a result of the ineffectiveness of selection in causing genetic fixation and its tendency to determine increased epistasis.

V. References

Bateman, K. G. (1956).—Studies in genetic assimilation. *Proc. R. Phys. Soc. Edin.* **25**: 1–6.

Dun, R. B. (1958).—Growth of the mouse coat. VI. Distribution and number of vibrissae in the house mouse. *Aust. J. Biol. Sci.* **11**: 95–105.

Dun, R. B. (1959*a*).—Ph.D. Thesis, University of Sydney.

Dun, R. B. (1959*b*).—The development and growth of vibrissae in the house mouse with particular reference to the time of action of the tabby (*Ta*) and ragged (*Ra*) genes. *Aust. J. Biol. Sci.* **12**: 312–30.

Dun, R. B., and Fraser, A. S. (1958).—Selection for an invariant character—"vibrissa number"—in the house mouse. *Nature* **181**: 1018–19.

Falconer, D. S. (1953).—Total sex linkage in the house mouse. *Z. indukt. Abstamm.-u. VererbLehre* **85**: 210–19.

Fisher, R. A. (1946).—"Statistical Methods for Research Workers." 10th Ed. (Oliver & Boyd: London.)

Fraser, A. S. (1959).—"Symposium of Biometrical Genetics." (In press.) (Pergamon Press: New York.)

GOLDSCHMIDT, R. (1935).—Gen und Ausseneigenschaft (Untersuchungen an *Drosophila*). *Z. indukt. Abstamm.-u. VererbLehre* **69**: 38–131.

LANDAUER, W. (1958).—On phenocopies, their developmental physiology and genetic meaning. *Amer. Nat.* **92**: 201–13.

LERNER, L. M. (1954).—"Genetic Homeostasis." (John Wiley & Sons, Inc.: New York.)

RENDEL, J. M. (1959).—The canalization of the scute phenotype. *Evolution* (in press).

WADDINGTON, C. H. (1953).—Genetic assimilation of an acquired character. *Evolution* **7**: 118–26.

WADDINGTON, C. H. (1956).—Genetic assimilation of bithorax phenotype. *Evolution* **10**: 1–13.

VARIATION AND DOMINANCE AT THE SCUTE LOCUS IN *DROSOPHILA MELANOGASTER*

By J. M. Rendel*

[*Manuscript received July* 16, 1959]

Summary

A genotypic scale, which is additive, has been worked out for variation of scutellar bristle number in *D. melanogaster*. sc^+ is shown to be partially dominant to sc^{sc}, the first substitution of sc^{sc} for sc^+ having about six times more effect on genotypic value than the second. Canalization at four bristles is responsible for complete dominance of sc^+ in stocks whose mean genotypic value gives a mean phenotype of four bristles in sc^+ flies. A function converting the phenotypic to the genotypic scale is given which could be used in heritability analysis.

I. Introduction

Selection for high scutellar bristle number in a *Drosophila melanogaster* stock segregating for sc^{sc} was found to be successful in an experiment already reported (Rendel 1959). Matings were between sc^{sc} males, chosen for high bristle number, and heterozygous females taken at random, all of which had four bristles in the early part of the experiment. Selection eventually resulted not only in an increase of the number of bristles on scute flies but also of the number on heterozygous scute females; but whereas the phenotype in scute flies was increased by about two bristles the corresponding change in heterozygous females was only a fraction of a bristle. It was shown by a method of analysis described below, that the estimated gene dose required to produce a given phenotypic change was much greater for some levels of phenotype than others. In Waddington's (1957) terminology, the four-bristle phenotype was strongly canalized and gene doses which produced large changes of phenotype at other levels of expression had no effect on phenotype at the level of four bristles. It was possible to map out a scale relating genotypic value to phenotypic change at different levels of expression over much of the phenotypic scale from 0 to 5 bristles; but the actual width of the four-bristle zone could be only approximately estimated. In the experiment now reported the gaps have been filled in from 0 to 7 bristles and it is possible to show the relative phenotypic effect of changes in genotypic value over this scale; the effect of a gene substitution of sc^+ for sc^{sc} in males and one or two sc^+ genes for sc^{sc} in females can also be demonstrated. Thus the dominance of sc^+ over sc^{sc} can be subdivided into the effect of gene substitution on genotypic value and the effect of changes in genotypic value on phenotype.

II. Treatment of the Data

Bristle number has been plotted against frequency of flies with a given bristle number in Figure 1. Frequencies have been converted to percentages. Three distributions are shown; these depict typical populations of sc^{sc} males from a line selected

---

* Animal Genetics Section, C.S.I.R.O., University of Sydney.

for low bristle number, sc^{sc}/sc^{sc} females from a line selected for high bristle number, and sc^{+}/sc^{+} females from the same line. These distributions of phenotype give an incomplete picture of the underlying genotypic variation. sc^{sc}/sc^{sc} females of the high line cover the one-, two-, and three-bristle classes quite well, but the four class is a threshold beyond which no further change in phenotype can be seen. The four class therefore contains all flies with a sufficiently high genotypic value to have at least four bristles; but, as no flies with five bristles appear in these sc^{sc}/sc^{sc} females, it is not possible to see directly from the graph of phenotypes how

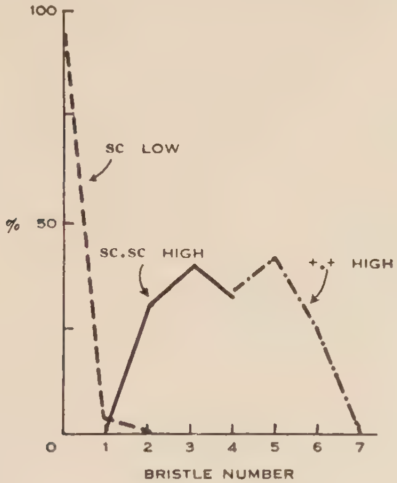


Fig. 1

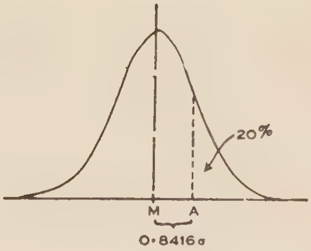


Fig. 2

Fig. 1.—Distribution of bristle number in sc^{sc} (low), sc^{sc}/sc^{sc} (high), and sc^{+}/sc^{+} (high). Subscripts to genotypes omitted for simplicity.

Fig. 2.—Normal distribution to illustrate estimation of distance between cut-off point (A) and mean (M) by taking probits.

genotypic values above four are distributed. The sc^{+}/sc^{+} females cover the range from five to seven bristles; but again the four class is a threshold and nothing can be seen directly, from the distribution of phenotypes, about genotypic values below the four threshold. The sc^{sc} males from the low-selection line are even more extreme; this genotype only has a few representatives with one and very few with two bristles, the rest all have none so that only a small tail of the distribution is revealed above the threshold of the zero-bristle class.

It will be realized that each class has two thresholds, an upper and a lower one; the four class has one boundary between four and five bristles and one between three and four. The fact that in the high line sc^{+}/sc^{+} females never have three bristles, and in the low line never have five, indicates that a very large genotypic value separates the top of the three class from the bottom of the five class and that the four class is an extremely broad one.

Although the phenotypic distributions reveal only part of the underlying genotypic variability, it is nevertheless possible to deduce something about the whole distribution of genotypic values from that part which is mirrored by the phenotype. If we have a distribution, some known percentage of which falls beyond a certain value as in Figure 2, conversion of the percentage into probits tells us how far the mean of the distribution is from the cut-off point in standard deviations. Thus if the point A cuts off 20 per cent. of the population, the distance between the mean, M, and A is 0·8416 standard deviations This can be looked up in tables of probits (cf. Fisher and Yates 1953). As the distributions of bristle numbers of various major genotypes in the up and down lines have several cut-off points, it is possible to map the distance between the cut-off points in standard deviations of the distribution covering them. For example, in Figure 1, 95·8 per cent. of scute males in the low line have no bristles; this corresponds to a probit of 1·73; that is to say, the mean of the distribution lies 1·73 σ below the lower boundary of the one class. 99·62 per cent. of the scute males lie below the lower boundary of the two class, that is to say have 0 or 1 bristle. This puts the mean of the distribution 2·67 σ below the lower boundary of the two class. By subtraction we find that the one class spans 0·94 σ since it runs from a point 2·67 σ above the mean to a point 1·73 σ above the mean. We cannot estimate the spread of the two class from this distribution because there were no flies with three bristles and therefore no cut-off point at the top of the two class. However, different genotypes span different parts of the phenotypic scale and by putting the results together the whole phenotypic range from 0 to 4 and 4 to 7 can be filled in.

The assumption which makes this possible is that the distribution about the mean at different phenotypes is the same. This distribution is basically the distribution of genotypic values and on theoretical grounds would not be expected to change much with selection in the absence of inbreeding. The observed phenotypic distribution does contain an environmental fraction but provided a further assumption can be made, that there are no gene environmental interactions which alter the percentage of genotypic values falling beyond a given threshold, without altering the mean accordingly, values estimated at different phenotypic levels will still be comparable. The justification of these assumptions is that where distributions overlap, whether they be of males or females, scute or wild type, high- or low-selection lines, they give estimates of the distance covered by a phenotypic class which are in very close agreement.

III. EXPERIMENTAL PROCEDURE

Two lines of flies were used in this experiment. The first consisted of a stock segregating for $sc^{sc}w^{w}$ and $sc^{+}w^{bl}$. Matings were always made between $sc^{sc}w^{w}/sc^{+}w^{bl}$ ♀♀ and either $sc^{sc}w^{w}$ or $sc^{+}w^{bl}$ ♂♂. Homozygous w^{bl}/w^{bl} is distinguishable from heterozygous w^{bl}/w^{w} which is distinguishable from w^{w}/w^{w}. In addition sc^{sc}/sc^{sc} can always be distinguished from sc^{sc}/sc^{+} or sc^{+}/sc^{+} by the number of abdominal chaetae. It is not possible to distinguish sc^{sc}/sc^{+} from sc^{+}/sc^{+} in this way except on averages. With the aid of these two scores, sc^{sc}/sc^{sc}, sc^{sc}/sc^{+}, and sc^{+}/sc^{+} genotypes can be distinguished with accuracy even when sc^{sc}/sc^{sc} flies have four scutellar bristles. Crossing over between sc and w is sufficiently rare not to introduce confusion into the scoring. This line was selected for high scutellar bristle number and when bristle number had increased sufficiently $sc^{sc}w^{w}/sc^{+}w^{bl}$ ♀♀ were mated to their $sc^{sc}w^{w}$ and $sc^{+}w^{bl}$ sibs in pairs. These matings gave rise to cultures segregating for sc^{sc}/sc^{sc} and sc^{sc}/sc^{+} on the one hand and sc^{sc}/sc^{+} and sc^{+}/sc^{+} on the other. A proportion of sc^{+}/sc^{sc} flies in this line had more than four bristles. A second line was similar except that w^{w} was in coupling with sc^{+} and w^{bl} with sc^{sc}. This line was selected for low scutellar bristle number. Response to selection became very slow as selection differential fell with drop in bristle number and in order to reduce bristle number far enough for some sc^{sc}/sc^{+} flies to have three bristles the final crosses were reared at 30°C instead of 25°C, high temperatures being known to reduce scutellar bristle number in scute stocks whereas low temperatures increase it. In order to test the possibility that the w^{bl} and w^{w} genes might differ in their effect on bristle number a small experiment was set up in which $sc^{sc}w^{w}$ and $sc^{sc}w^{bl}$ were segregating. There was no difference in the scutellar bristle number of w^{bl} and w^{w} sibs. This was felt to be important not so much when comparing the high

TABLE 1

BRISTLE NUMBER OF DIFFERENT GENOTYPES IN HIGH AND LOW LINES IN ABSOLUTE NUMBERS, CONVERTED TO PERCENTAGES AND TO PROBITS

(*a*) Bristle number of different genotypes

Genotype	Bristle Score	High Line		Total	Low Line		Total
		× sc^{sc}	× sc^{+}		× sc^{sc}	× sc^{+}	
sc^{sc}	0	1		1	128	125	253
	1	7	2	9	5	5	10
	2	75	53	128		1	1
	3	65	69	134			
	4	31	50	81			
sc^{+}	4	110	131	241	158	159	317
	5	31	58	89			
	6	6	8	14			
$sc^{+}sc^{sc}$	3				2	2	4
	4	144	141	285	162	152	314
	5	27	48	75			
	6	2	5	7			
$sc^{sc}sc^{sc}$	0					123	123
	1	1		1			
	2	38		38		7	7
	3	72		72			
	4	52		52			
$sc^{+}sc^{+}$	4		48	48	194		194
	5		61	61			
	6		37	37			
	7		1	1			

TABLE 1 (*Continued*)

(*b*) Bristle number converted to percentages and probits

Genotype	Bristle Score	High Line (in accumulated %)	Probits (from mean)	Low Line (in accumulated %)	Probits (from mean)
sc^{sc}	0	0·28	−2·77	95·8	+1·73
	1	2·83	−1·90	99·6	+2·65
	2	39·1	−0·28	100	
	3	77·1	+0·74		
	4	100			
sc^{+}	4	70·1	+0·53	100	
	5	95·9	1·74		
	6	100			
$sc^{+}sc^{sc}$	3			1·26	−2·24
	4	77·7	+0·76	100	
	5	98·1	+2·07		
	6	100			
$sc^{sc}sc^{sc}$	0			81·5	+0·90
	1	0·61	−2·50	95·4	+1·68
	2	23·9	−0·71	100	
	3	68·1	+0·47		
	4	100			
$sc^{+}sc^{+}$	4	32·7	−0·45	100	
	5	74·1	+0·65		
	6	99·3	+2·46		
	7	100			

and low lines as when comparing homozygous and heterozygous females of the same lines for dominance of sc^{+} over sc^{sc} since the comparison is between $sc^{sc}w^{w}/sc^{sc}w^{w}$, $sc^{sc}w^{w}/sc^{+}w^{bl}$, and $sc^{+}w^{bl}/sc^{+}w^{bl}$, or vice versa depending on the genes in coupling.

IV. RESULTS

The scores of flies in the two types of cross in the high line and the two types of cross in the low line are set out at the top of Table 1. The two types of cross in each case have been summed wherever they contain identical major genotypes. The totalled scores have been converted to percentages in the bottom half of the table and the probits, measured from the mean of the distributions, are shown next to the percentages. From these probit values it is possible to estimate the distance in standard deviations covered by the 1-, 2-, 3-, 5-, and 6-bristle classes. In Table 2 three estimates of the one class have been given and averaged, these come from sc^{sc} ♂♂ of the high and low lines and sc^{sc}/sc^{sc} ♀♀ of the low line; the same procedure has been followed for the remaining bristle classes. By comparing sc^{sc} ♂♂ and

sc^{sc}/sc^{sc} ♀♀ of the high and low lines, three estimates of the difference between the lines have been obtained and averaged. These are the distances between the 0/1 cut-off point and the 1/2 cut-off point in males of the two lines and the distance between the 1/2 cut-off point in females. The average difference between lines is 4·41 σ. If we assume the two distributions of heterozygotes are separated by this distance, the distance covered by the four-bristle class is given by adding 4·41 to the distance of the mean of the low line sc^{+}/sc^{+} females from the bottom four boundary, 2·24 σ, and the distance of the high line sc^{sc}/sc^{+} ♀♀ from the top four boundary, 0·76 σ. The distance between sc^{sc}/sc^{sc} and sc^{+}/sc^{sc}, sc^{+}/sc^{sc} and sc^{+}/sc^{+}, and sc^{sc} and sc^{+} in the high line can be estimated directly. In the low line the position of the sc^{+} and sc^{+}/sc^{+} means must first be estimated from the position of equivalent classes in the high line by subtracting 4·41, the distance between lines. It is then possible, in addition, to estimate distances between sexes. The complete picture is shown in Table 2 and Figure 3. The genotypic values shown in Figure 3 are plotted against phenotypic means in Figure 4. The curve in Figure 4 fits, approximately, the expression

TABLE 2

ESTIMATION OF DISTANCES IN STANDARD DEVIATIONS OF THE DISTRIBUTION OF BRISTLE NUMBER, THIS DISTRIBUTION BEING ASSUMED CONSTANT OVER ALL PHENOTYPES

	Estimates of Distance (σ)	Sum or Average
Bristle class		
1	0·87, 0·92, 0·78	0·86
2	1·62, 1·79	1·70
3	1·02, 1·18	1·10
4	2·24 + 4·41 + 0·76	7·41
5	1·21, 1·31, 1·11	1·21
6		1·81
High → low	4·50, 4·55, 4·18	4·41
High line		
$sc^{sc}/sc^{sc} \rightarrow sc^{sc}/sc^{+}$	0·47 − 0·76 + 7·41	7·12
$sc^{+}/sc^{sc} \rightarrow sc^{+}/sc^{+}$	0·76 + 0·45	1·21
$sc^{sc} \rightarrow sc^{+}$	−0·53 + 7·41 + 0·74	7·62
Low line		
$sc^{sc}/sc^{sc} \rightarrow sc^{sc}/sc^{+}$	0·90 + 0·86 + 1·70 + 1·10 + 2·24	6·80
$sc^{+}/sc^{sc} \rightarrow sc^{+}/sc^{+}$	7·41 − 2·24 − 4·41 + 0·45	1·21
$sc^{sc} \rightarrow sc^{+}$	7·41 − 4·41 − 0·53 + 1·73 + 3·66	7·86

$$Y = -2{\cdot}365 + 5{\cdot}4196x - 2{\cdot}2759x^{2} + 0{\cdot}05383x^{3} + 0{\cdot}1675x^{4} - 0{\cdot}021467x^{5}$$

up to $x = 5$. This expression was calculated from the points plotted on the University of Sydney's electronic computer, SILLIAC.

V. Discussion

This investigation is one illustration of the general problem of scaling data for biometrical analysis, a subject which is discussed by Mather (1949) in his book "Biometrical Genetics". In this attempt at scaling the object has been to determine directly what equal changes of genotypic value do to phenotype over the whole range of phenotypes from 0 to 7 bristles, rather than find by trial and error some function which appears to reduce variance. The scale is successful insofar as substitutions of sc^+ for sc^{sc} have the same effect over the whole scale. The validity of the scaling method depends on assumptions already dealt with in Section II.

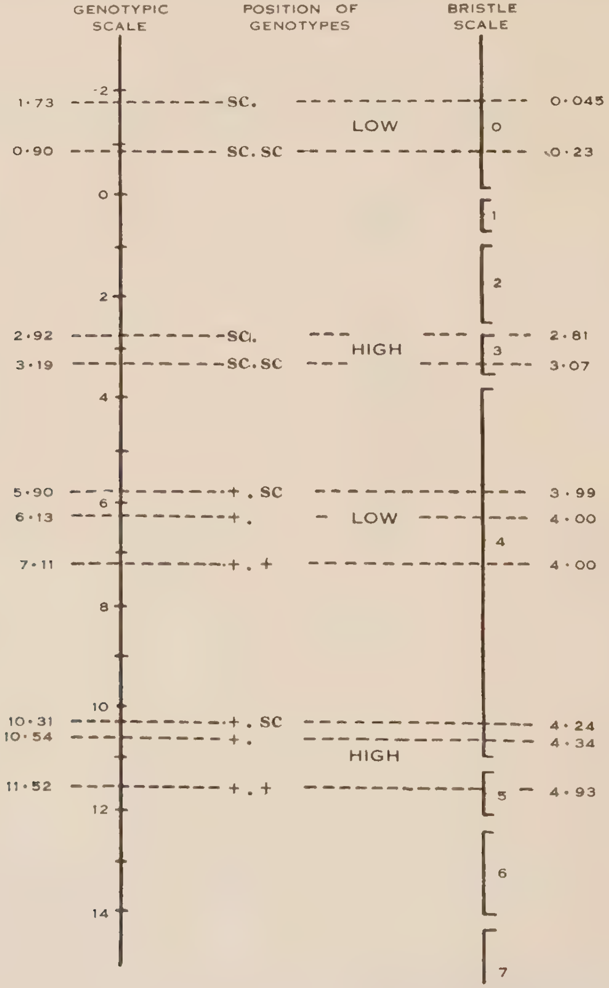


Fig. 3.—Chart showing genotypic scale on the left, phenotypic scale on the right. Position of different genotypes and lines is shown on both scales. Numbers on the extreme left are standard deviations from 0. Numbers on the extreme right are mean bristle numbers. Subscripts to genotypes omitted for simplicity.

Part of the inadequacy of the bristle score as a scale might be overcome by measuring the length and diameter of bristles in addition to counting them. In the low line sc^+/sc^{sc} flies had noticeably smaller bristles than their sc^+/sc^+ sibs and in some few flies the "bristle" was only a bristle site or a thin short wisp of bristle. However, all such had been defined before the experiment as bristles and so were scored as such. Detailed examination of these, particularly at the edges of the four-bristle class is a subject for future investigation.

The final scale has a most marked broadening of the four class and smaller ones at the two and six classes. The symmetry of the scale about four is intriguing but may have no significance. The character is certainly markedly canalized.

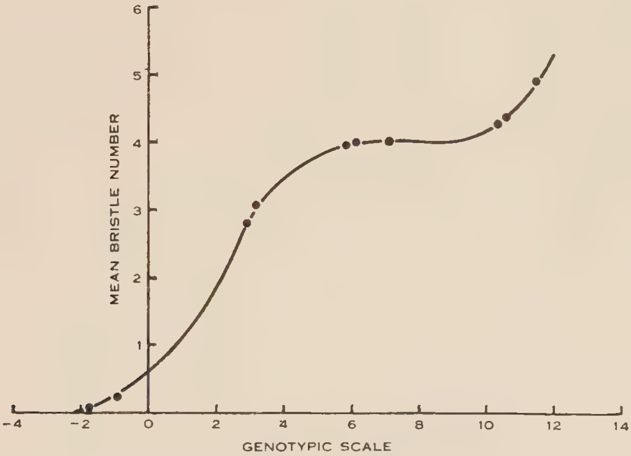


Fig. 4.—Phenotypic change plotted against change measured on the genotypic scale taken from Figure 3.

There is, of course, one assumption not so far mentioned, which has been made throughout the analyses; it is assumed that the genes determining the canalization zone, or the relationship of genotypic value to phenotype, do not alter significantly throughout the experiment. It has been shown elsewhere that this relationship can be altered by appropriate selection; but in the work reported here no such selection was applied and changes in the mean expression of the character, as for example between scute and non-scute phenotypes or high- and low-selection lines, are very large compared to changes, which can be brought about by selection, in the width of a canalization zone. The idea is that genotypic values are completely additive, but changes in genotypic values have effects on phenotype depending on the level of expression of the character; this gives rise to epistatic effects near the canalization zone. Further epistatic effects would result from gene variation changing the degree of canalization.

Since on the phenotypic scale the effect of a gene substitution depends on phenotype and this is determined not only by other genes but at least by temperature and possibly crowding also, there will be both epistatic and gene environment interactions on this scale. However, they will not be specific to particular genes and particular environments and can be removed by conversion to the genotypic scale by using the expression $Y = (F)X$. Heritabilities calculated on the genotypic scale should give valid predictions over the whole range of scutellar bristle number so far examined.

Dominance of sc^+ over sc^{sc} is shown up very clearly. In unselected stocks a single sc^+ gene puts the mean phenotype near the middle of the four zone and this is so broad that no effect is gained by adding another sc^+ gene. However, if the mean of the population is increased or decreased sufficiently, effects of a second sc^+ gene substitution can be measured. Dominance of sc^+ is largely due to the fact that the first gene substitution has a genotypic value of about 7 whereas the second has one of only 1·21. But complete dominance is brought about when the heterozygote has a mean genotypic value at least $2 \cdot 5\ \sigma$ above the lower boundary of the four class and at least $3 \cdot 5\ \sigma$ below the upper boundary. If it is closer to either boundary a count of sufficient flies will show some effect of the second substitution. It seems probable that selection of modifiers in at least some cases will affect dominance by creating a zone of canalization resistant to all forms of variation. On the other hand, it is hard to see how the difference in genotypic value of the first and second gene substitution can be due to anything but some form of chemical equilibrium analogous to pH buffering and other mass action equilibria. If, for example, the rate of a reaction leading to bristle formation were dependent on the rate of removal of end-product, a second sc^+ gene would have an effect only insofar as removal of end-product was determined by its rate of formation and insofar as two genes would reach equilibrium sooner than one. This is in contrast to the dominance found by Dun and Fraser for the tabby gene in mice (Dun and Fraser 1959). Heterozygotes appear to have an intermediate genotypic value and all dominance comes from canalization. Presumably the Ta^+ gene has no limit to its action other than its own efficiency.

The type of dominance for sc^+ is of particular interest in the light of Haldane's discussion of industrial melanism (Haldane 1957). Haldane shows there is not time for melanism to acquire dominance through differential survival of genes modifying specifically the dominance of a melanic gene in the heterozygote; but if melanism is of advantage and is initially variable in the homozygous as well as the heterozygous state in response to both genetic and environmental change, there will be a tendency for canalization of the character to appear as a result of differential survival in homozygotes and with this a great reinforcement to other forms of dominance.

VI. Acknowledgment

I am indebted to Dr. A. S. Fraser for preparing the figures and for calculating the regression equation.

VII. References

Dun, R. B., and Fraser, A. S. (1959).—Selection for an invariant character, vibrissa number, in the house mouse. *Aust. J. Biol. Sci.* **12**(4): 506–23.

FISHER, R. A., and YATES, F. (1953).—"Statistical Tables for Biological, Agricultural, and Medical Research." 4th Ed. (Oliver and Boyd: Edinburgh.)

HALDANE, J. B. (1957).—The cost of natural selection. *J. Genet.* **55**: 511–24.

MATHER, K. (1949).—"Biometrical Genetics." (Methuen: London.)

RENDEL, J. M. (1959).—Canalization of the scute phenotype. *Evolution* (in press).

WADDINGTON, C. H. (1957).—"Strategy of the Genes." (Allen and Unwin: London.)

SHORT COMMUNICATIONS

THE USE OF THYROXINE IN THE BIOASSAY OF GROWTH HORMONE*

By A. L. Wallace†

Hypophysectomy causes inactivity of the thyroid gland and it has been known for some time that thyroxine augments the body weight and skeletal growth responses to growth hormone in hypophysectomized and hypophysectomized–thyroidectomized rats (Smith 1933; Evans, Simpson, and Pencharz 1939; Scow *et al*. 1949; Li 1953; Asling *et al*. 1954).

This effect has been used by Geschwind and Li (1952) to obtain increased response to growth hormone in the tibial assay by simultaneously giving thyroxine. However, they did not suggest its use as a routine assay procedure.

Bülbring (1938), using increase in body weight in hypophysectomized rats as an index of response to growth hormone, observed that rats given a second series of growth hormone injections did not respond as well as they had to the first series of injections. Evans *et al*. (1938) observed a similar phenomenon and also remarked on the declining sensitivity to growth hormone with increase in age after hypophysectomy.

We ourselves have observed both these effects in the routine assay of growth hormone in hypophysectomized rats. In fact, if our particular strain of rats is used later than about one month after hypophysectomy a large proportion of them are completely refractory. In an attempt to overcome these difficulties and to improve the sensitivity of the assay we have investigated the routine use of thyroxine in growth hormone assays based on the increase in body weight of hypophysectomized rats.

Methods

Albino rats were hypophysectomized at 6 weeks of age and kept at 80°F. A pelleted diet and fresh milk were fed *ad lib*. All injections were given intraperitoneally. Rats were weighed daily to the nearest gram.

The regression of body weight on time was calculated and the response to growth hormone treatment expressed as the rate of change in body weight in g per day. The regression coefficient is more accurate than the customary method of taking the difference between the first and final body weights of the treatment period.

---

* Manuscript received May 26, 1959.

† Division of Animal Health and Production, C.S.I.R.O., Sheep Biology Laboratory, Prospect, N.S.W.

Results

Experiment 1.—Forty-eight rats hypophysectomized 3–4½ months previously were divided into six groups. All rats were injected with a sheep growth hormone preparation [56·17 (3); IV], half with a dose of 80 μg and half with a dose of 320 μg daily for 10 days. At each growth hormone dosage level, different groups received doses of 0, 2, or 4 μg of sodium L-thyroxine daily. The results are plotted in Figure 1. A positive dose response curve was not obtained in the absence of thyroxine. A dose of 2 μg of thyroxine enhanced the response to increased growth hormone dose as much as a dose of 4 μg.

Experiment 2.—Forty-eight rats, hypophysectomized 1½–2 months previously, were divided into four groups. These groups were used to assay two different homogenate preparations (A and B) from sheep pituitary glands. Each preparation yielded a particle extract (P) and a supernatant (S) thus giving four fractions, each of which was assayed at two dosage levels.

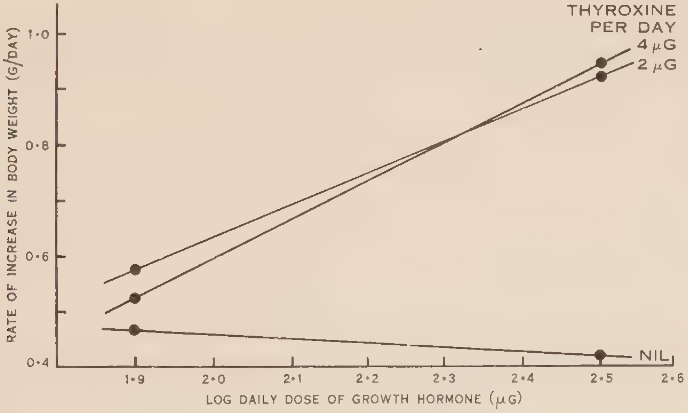


Fig. 1.—Effect of thyroxine on the body weight response to a sheep growth hormone preparation.

Two weeks after the conclusion of this assay the same forty-eight rats were re-randomized and a similar assay was carried out on them, only this time 2 μg of thyroxine was also given daily to each rat. These results are given in Figure 2 and show that consistent dose response curves were obtained only when thyroxine was used. In this experiment when no thyroxine was given 24 of the 48 rats were completely refractory. In the second assay when thyroxine was given only six rats failed to respond. Of these two were obviously sick and declined in body weight throughout treatment, dying shortly after treatment ceased.

Experiment 3.—Forty-five rats, hypophysectomized 1–1½ months previously, were used in a third experiment. A growth hormone preparation kindly donated by the Endocrinology Study Section, National Institute of Health (Somar-A, bovine, lot No. R 50109, prepared by the Armour Laboratories, Illinois, U.S.A.), and a sheep pituitary preparation [57·9 (12) 20i] were compared at two dosage levels. There were nine rats at each dosage level, five of which received 2 μg thyroxine daily.

The remaining nine rats were used as a control group and five of these also received 2 μg thyroxine. The results of this experiment are shown in Figure 3. The dose response curves in the absence of thyroxine were very flat. Thyroxine enhanced the response to both growth hormone preparations. Thyroxine alone did not produce any growth in the hypophysectomized rats.

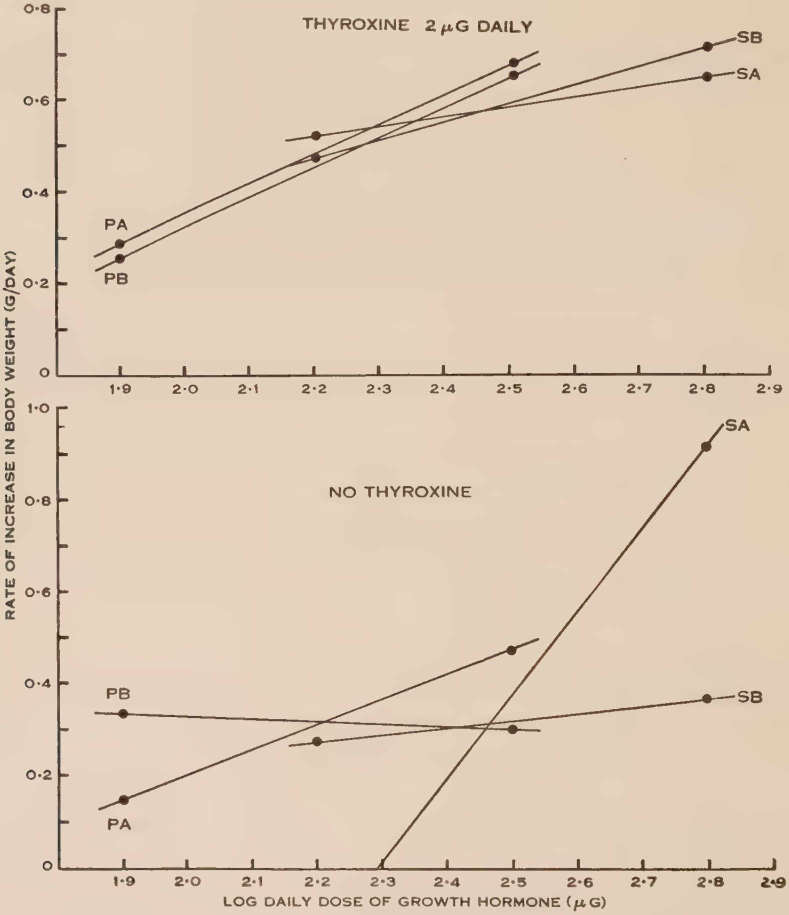


Fig. 2.—Effect of thyroxine on the body weight response to two sheep pituitary fractions. *PA*, *SA*, particle and supernatant fractions of preparation A; *PB*, *SB*, particle and supernatant fractions of preparation B.

Discussion

The results clearly show the advantage of injecting thyroxine when our particular strain of rats is used to assay growth hormone by the body-weight-increase method. It is reasonable to assume that thyroxine would be of benefit in reducing variation between individuals in most strains of rats for it is probable that some of this variation is due to differences in residual thyroid activity.

It appears that low thyroid activity is not the only factor responsible for decreased sensitivity to growth hormone for even in groups given thyroxine there were still a few refractory rats that did not respond at all to growth hormone. It is possible that the degree of residual adrenal function is also important in determining the response to growth hormone. Not much data are available on this point. Geschwind and Li (1954) have shown that deoxycorticosterone acetate, 1 mg/day, in the hypophysectomized–adrenalectomized rat permits a normal tibial response to growth hormone injections.

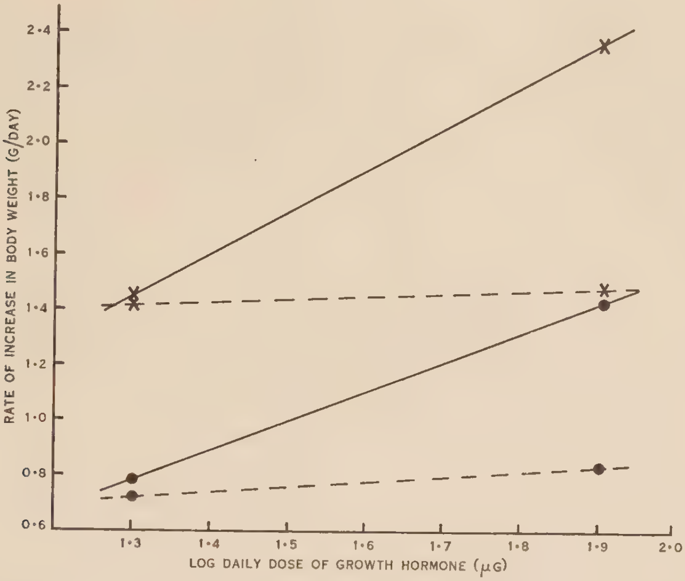


Fig. 3.—Effect of thyroxine on the body weight response to standard ox growth hormone (×) and to a sheep growth hormone (●) preparation. ———— 2 μg thyroxine; – – – – no thyroxine.

One other possible benefit from the use of thyroxine is in preventing an inflated response to growth hormone due to contamination of a preparation with thyroid-stimulating hormone. Geschwind and Li (1954) have suggested that in the tibial assay only very large doses of thyroid-stimulating hormone augment the response to growth hormone, but in assays using body weight gain as an index of growth hormone activity the longer injection period may enable a greater response to thyroid-stimulating hormone.

References

Asling, C. W., Simpson, M. E., Li, C. H., and Evans, H. M. (1954).—*Anat. Rec.* **119**: 101.

Bülbring, E. (1938).—*Quart. J. Pharm.* **11**: 26.

Evans, H. M., Simpson, M. E., and Pencharz, R. I. (1939).—*Endocrinology* **25**: 175.

Evans, H. M., Uyei, N., Bartz, Q. R., and Simpson, M. E. (1938).—*Endocrinology* **22**: 483.

Geschwind, I. I., and Li, C. H. (1952).—*J. Clin. Endocrinol.* **12**: 937.

Geschwind, I. I., and Li, C. H. (1954).—In "Hypophyseal Growth Hormone, Nature and Actions". p. 28. International Symposium. (Eds. R. W. Smith, O. H. Gaebler, and C. N. Long.) (Henry Ford Hospital: Detroit.)

Li, C. H. (1953).—"Ciba Foundation Colloquia on Endocrinology." Vol. 5. p. 115.

Scow, R. O., Simpson, M. E., Asling, C. W., Li, C. H., and Evans, H. M. (1949).—*Anat. Rec.* **104**: 445.

Smith, D. E. (1933).—*Proc. Soc. Exp. Biol., N.Y.* **30**: 1252.

STUDIES OF CASEIN

IV. THE ISOLATION OF κ-CASEIN*

By R. G. Wake†

The preparation of second-cycle casein—fraction S—from skim milk has been described in Part I of this series (McKenzie and Wake 1959*a*) and by Waugh and von Hippel (1956). This fraction contains mainly κ-casein, as well as β- and γ-casein, and a number of minor components. In the present communication a method is described for the separation and purification of the κ-casein. Some observations on its chemical and physical properties are also included.

Experimental

A solution of second-cycle casein—fraction S (Fig. 1(*a*)) was prepared from skim milk according to the earlier procedure except for centrifugation at 50,000 r.p.m. (using a Sharples supercentrifuge with a type T-9-46 clarifier bowl) to spin out and wash the micelles. The pH of this solution (containing approx. 0·3 per cent. protein) was adjusted to pH 4·6 at 2°C with 0·5N HCl. On subsequent warming to 35°C most of the protein was precipitated. After collecting the precipitate by centrifugation for 20 min at 1000 *g* and room temperature in a M.S.E. laboratory centrifuge, it was dissolved in water at pH 7·5 by the slow addition of 1N NH_3 to give a 2 per cent. solution approximately. This was made 0·4M with ammonium acetate, and 95 per cent. ethanol was added slowly, with stirring, to a final concentration of 50 per cent. The solution was adjusted to pH 5·7 (see Part I for pH measurements) by the slow addition of 2N acetic acid in 50 per cent. ethanol. Stirring was continued for 1 hr and the precipitate removed by filtration on a Whatman No. 1 paper. This material contained approximately 90 per cent. κ-casein (Fig. 1(*b*)). Most of the slowly sedimenting impurity, probably β-casein, was removed as follows: the crude κ-casein was dissolved at pH 12 by the slow addition of 2N NaOH to give a 0·4 per cent. solution approximately, adjusted to pH 7 with 1N HCl, cooled to 2°C, and finally precipitated at pH 4·4 by the addition of cold 0·2N HCl (β-casein is more soluble than κ-casein under these conditions of temperature and pH). The precipitate was obtained by centrifugation for 10 min at 2°C and 895 *g* in an International type PR-2 refrigerated centrifuge. It was essentially pure κ-casein with only a trace, approximately 2 per cent., of β-casein, (Fig. 1(*c*)). Attempts to remove this impurity, which could only just be detected on paper electrophoresis, by dissolution to 0·2 per cent. protein concentration and precipitation at pH 4·4 and 2°C were unsuccessful. This acid-precipitated κ-casein was dissolved at pH 12 (the material is soluble with difficulty at lower pH), adjusted to pH 7, and freeze-dried. Approximately 3 g κ-casein was obtained from 14 l. skim milk.

---

* Manuscript received January 30, 1959.

† Biochemistry Department, University of Sydney; present address: Department of Biochemistry, Stanford University, California.

Properties

Freeze-dried κ-casein was readily soluble in water at neutral pH and gave no precipitate on the addition of calcium chloride. With α-casein and in the presence of 0·06M $CaCl_2$ it gave micelles which could be clotted by rennin. Preliminary analyses have indicated the presence of phosphorus and neuraminic acid, as well as carbohydrate. No significant quantities of free α-amino end-groups could be detected in κ-casein by means of the fluorodinitrobenzene method of Sanger (1945). The possibility of arginine being present as an end-group has not been settled (see Part V, Wake 1959).

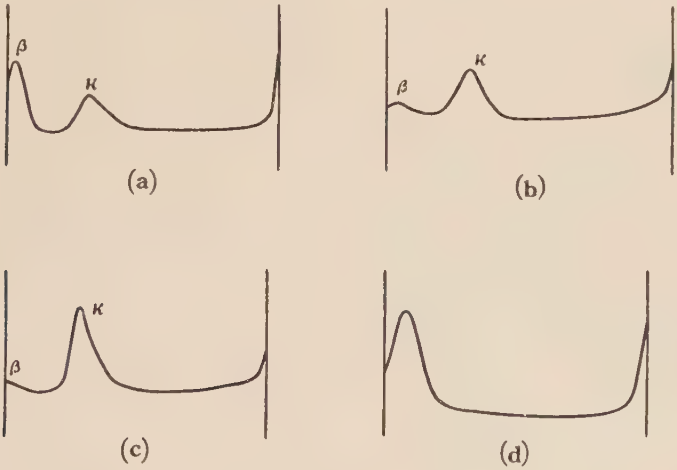


Fig. 1.—Sedimentation (at 59,780 r.p.m.) of fractions obtained during the purification of κ-casein. (*a*), (*b*), and (*c*) were carried out in sodium phosphate buffer, pH 7·0, ionic strength 0·1 (0·05M NaCl) at 2–5°C, using 0·5–1 per cent. protein: (*a*) second-cycle casein—fraction S, 34 min, $\theta = 65°$; (*b*) crude κ-casein, 36 min, $\theta = 55°$; (*c*) purified κ-casein, 26 min, $\theta = 60°$; (*d*) 1 per cent. κ-casein in sodium phosphate buffer, pH 12·0, ionic strength 0·19, at room temperature, 59 min, $\theta = 65°$. The preparation of the pH 7·0 buffer is described in Part I (McKenzie and Wake 1959*a*), and that of the pH 12·0 buffer in Part III (McKenzie and Wake 1959*b*).

Moving-boundary electrophoresis of 0·7 per cent. κ-casein in sodium phosphate buffer at pH 7·1, ionic strength 0·1 (0·08M NaCl), showed a single peak in both the ascending and descending limbs. The mobility was $-6{\cdot}80\times10^{-5}$ cm^2 V^{-1} sec^{-1}. Sedimentation under similar conditions (pH 7·0, low temperature) showed essentially a single peak with S_{20} approximately 13 S (Fig. 1(*c*)). This peak represents an aggregate of very high molecular weight. In sodium phosphate buffer at pH 12·0, ionic strength 0·19 (von Hippel and Waugh 1955), 1·0 per cent. κ-casein showed a single, well-defined peak, with $S_{20} = 1{\cdot}0$ S (Fig. 1(*d*)). This probably represents the κ-casein monomer.

An estimate of the molecular weight of κ-casein at pH 12·0 has been obtained by application of the Archibald ultracentrifugal procedure (see Part III, McKenzie and Wake 1959*b*). The molecular weight is in the vicinity of 26,000.

κ-casein can thus be prepared in essentially pure form from soluble casein. It is a mucoprotein containing phosphorus, neuraminic acid, and probably other sugars (cf. bovine submaxillary mucin (Gottschalk 1958)) and has no detectable α-amino end-groups in significant quantities. It is highly aggregated at neutral pH but shows single peaks on electrophoresis and sedimentation. It can be disaggregated into monomers at pH 12 with a molecular weight of approximately 26,000.

Acknowledgments

Grateful acknowledgment is due to Dr. H. A. McKenzie and Mr. M. B. Smith for their advice and assistance during the course of this work. The author is indebted to Dr. K. Knox for carrying out the carbohydrate analyses. Other acknowledgments are given in Part I.

References

GOTTSCHALK, A. (1958).—*Nature* **181**: 377.

HIPPEL, P. H. VON, and WAUGH, D. F. (1955).—*J. Amer. Chem. Soc.* **77**: 4311.

MCKENZIE, H. A., and WAKE, R. G. (1959*a*).—*Aust. J. Chem.* **12** (4) (in press).

MCKENZIE, H. A., and WAKE, R. G. (1959*b*).—*Aust. J. Chem.* **12** (4) (in press).

SANGER, F. (1945).—*Biochem. J.* **39**: 507.

WAKE, R. G. (1959).—*Aust. J. Biol. Sci.* **12**: 479.

WAUGH, D. F., and HIPPEL, P. H. VON (1956).—*J. Amer. Chem. Soc.* **79**: 4576.

INDEX

PAGE

Action Potentials, Studies of, in the Vacuole and Cytoplasm of *Nitella* 412

Aerobacter aerogenes, Adsorption by, of *p*-Hydroxybenzoic Acid and its Esters and of Sodium Benzoate at Different pH Levels .. 204

Amino Acids, Free, of Cabbage and Cauliflower Plants, Effect of Chloride on 160

Anguina agrostis, Influence of the Soil Moisture Regime on the Expulsion of the Larval Mass of the Nematode, from Galls .. 247

Antiauxin, Extension Growth Activities of some *cyclo*Propane Derivatives, a New Class of .. 257

Apple Fruit, Oxidation of Krebs Cycle Acids by Tissue Slices and Cytoplasmic Particles from .. 167

Apples, Studies in the Natural Coating of. IV 175

Arabidopsis thaliana, a Study of High Temperature Lesions in .. 117

Auxin, Residual Effects of, in Cell Extension 373

Auxins, Neutral and Acidic, in Developing Tobacco Fruit .. 340

Avocado Fruit, Chemical Changes during Growth and Storage of 445

Ballard, L. A. T., and Grant Lipp, A. E.—
Differential Specificity Exhibited by Two Germination Inhibitors Present in *Echium plantagineum* L. 343

PAGE

Barbour, R. G. H.—
The Adsorption by *Aerobacter aerogenes* of *p*-Hydroxybenzoic Acid and its Esters and of Sodium Benzoate at Different pH Levels 204

Barley Kernel, Developing, Source of Carbon for 40

Barley Kernel, Developing, Starch Granule Formation in 146

Blake, C. D.—
See Collis-George, N. 247

Boophilus microplus, Studies on "Haemixodovin", the Pigment in the Eggs of 263

Bos taurus L., Pre-natal Development of Skin and Hair in .. 72

Bouma, D.—
See Groenewegen, H. 16

Braden, A. W. H.—
Strain Differences in the Morphology of the Gametes of the Mouse 65

Bremner, K. C.—
Studies on "Haemixodovin", the Pigment in the Eggs of the Cattle Tick *Boophilus microplus* (Acarina: Ixodidae) 263

Buttrose, M. S., and May, C. H.—
Physiology of Cereal Grain. I. The Source of Carbon for the Developing Barley Kernel .. 40

Buttrose, M. S. —
See also May, L. H. 146

Cabbage and Cauliflower Plants, Effect of Chloride on the Free Amino Acids of 160

PAGE

Calcium in the Nitrogen Metabolism of Subterranean Clover 26

Calcium Metabolism in the Bones of Young Rats, some Effects of Fluoride on 466

S-Carboxymethylkerateines and *S*-Sulphokerateines from Wool, Electrophoretic Properties of .. 96

Carr, D. J., and Ng, E. K.—
  Residual Effects of Auxin, Chelating Agents, and Metabolic Inhibitors in Cell Extension .. 373

Casein, Action of Rennin on .. 479

κ-Casein, Isolation of 538

Casein, Studies of. IV, V .. 538, 479

Cattle Tick, Studies on "Haemixodovin", the Pigment in the Eggs of the 263

Cauliflower and Cabbage Plants, Effect of Chloride on the Free Amino Acids of 160

Cereal Grain, Physiology of. I, II 40, 146

Chaetomium, Formation of the Colony in the Fungus 53

Chara australis, Ionic Relations of Cells of. I 395

Chara australis R. Br., Water Permeability of Cells of 136

Chelating Agents, Residual Effects of, in Cell Extension 373

Collis-George, N., and Blake, C. D.–
  The Influence of the Soil Moisture Regime on the Expulsion of the Larval Mass of the Nematode *Anguina agrostis* from Galls .. 247

Cutin, Nature of 175

Dainty, J., and Hope, A. B.—
  The Water Permeability of Cells of *Chara australis* R. Br. .. 136
  Ionic Relations of Cells of *Chara australis*. I. Ion Exchange in the Cell Wall 395

PAGE

Dart, P. J., and Pate, J. S.—
  Nodulation Studies in Legumes. III. The Effects of Delaying Inoculation on the Seedling Symbiosis of Barrel Medic, *Medicago tribuloides* Desr. 427

Davenport, J. B., and Ellis, S. C.—
  Chemical Changes during Growth and Storage of the Avocado Fruit 445

Delwiche, C. C. —
  See Freney, J. R. 160

DNP-Cysteic Acid, Method for the Isolation of 303

Drosophila melanogaster, Variation and Dominance at the Scute Locus in 524

Dun, R. B.—
  The Development and Growth of Vibrissae in the House Mouse with Particular Reference to the Time of Action of the Tabby (*Ta*) and Ragged (*Ra*) Genes 312

Dun, R. B., and Fraser, A. S.—
  Selection for an Invariant Character, Vibrissa Number, in the House Mouse 506

Dunstone, J. R., and Payne, E.—
  Some Effects of Fluoride on Calcium Metabolism in the Bones of Young Rats 466

Echium plantagineum L., Differential Specificity Exhibited by Two Germination Inhibitors Present in 343

Ellis, S. C.—
  See Davenport, J. B. 445

Findlay, G. P.—
  Studies of Action Potentials in the Vacuole and Cytoplasm of *Nitella* 412

PAGE

Flax Roots, Isolated, Potentiation of Lateral Root Induction by Root Initials in 388

Fluoride, some Effects of, on Calcium Metabolism in the Bones of Young Rats 466

Fraser, A. S., Nay, T., and Kindred, B.—
Variation of Vibrissa Number in the Mouse 331

Fraser, A. S.—
See also Dun, R. B. 506

Freney, J. R., Delwiche, C. C., and Johnson, C. M.—
The Effect of Chloride on the Free Amino Acids of Cabbage and Cauliflower Plants 160

Gametes of the Mouse, Strain Differences in the Morphology of 65

Gates, C. T.—
See Groenewegen, H. 16

Genetic Effects of Family Structure in Natural Populations 1

Genetical Effects of Population Subdivision, Theory of some . . 109

Genetic Sampling in a Random Mating Population of Constant Size and Sex Ratio 500

Germination Inhibitors Present in *Echium plantagineum* L., Differential Specificity Exhibited by Two 343

β-Glucosidases Secreted by Fungi, some Comparative Properties of 213

Goldacre, P. L.—
Potentiation of Lateral Root Induction by Root Initials in Isolated Flax Roots 388

Grant Lipp, A. E.—
See Ballard, L. A. T. 343

Griffing, B.—
See Langridge, J. 117

PAGE

Groenewegen, H., Bouma, D., and Gates, C. T.—
Uptake and Translocation of Chlorine in Citrus Cuttings during and after a Short Salt Treatment 16

Growth Hormone, Use of Thyroxine in the Bioassay of 534

"Haemixodovin", the Pigment in the Eggs of the Cattle Tick, Studies on 263

Hatch, M. D., Pearson, Judith A., Millerd, Adele, and Robertson, R. N.—
Oxidation of Krebs Cycle Acids by Tissue Slices and Cytoplasmic Particles from Apple Fruit . . 167

Heideman, Margaret J.—
See Lyne, A. G. 72

Hope, A. B.—
See Dainty, J. 136, 395

House Mouse, Development and Growth of Vibrissae in the, with Particular Reference to the Time of Action of the Tabby (*Ta*) and Ragged (*Ra*) Genes 312

House Mouse, Selection for an Invariant Character, Vibrissa Number, in the 506

Huelin, F. E.—
Studies in the Natural Coating of Apples. IV. The Nature of Cutin 175

Hypothyroidism in the Sheep . . 455

Jermyn, M. A.—
Some Comparative Properties of β-Glucosidases Secreted by Fungi 213

Johnson, C. M.—
See Freney, J. R. 160

Kefford, N. P.—
Extension-growth Activities of some *cyclo*Propane Derivatives, a New Class of Antiauxin . . 257

PAGE
Keratinolytic Bacillus, Digestion of Wool by a 274
Kindred, B.—
  See Fraser, A. S. 331
Krebs Cycle Acids, Oxidation of, by Tissue Slices and Cytoplasmic Particles from Apple Fruit .. 167

Lahovary, N.—
  See Mushin, Rose .. 223, 233
Langridge, J., and Griffing, B.—
  A Study of High Temperature Lesions in *Arabidopsis thaliana* 117
Lascelles, A. K., and Setchell, B. P.–
  Hypothyroidism in the Sheep .. 455
Lateral Root Induction, Potentiation of, by Root Initials in Isolated Flax Roots 388
Latter, B. D. H.—
  Genetic Sampling in a Random Mating Population of Constant Size and Sex Ratio 500
Legumes, Nodulation Studies in. III 427
Lilienthal, B.—
  See Nicholson, R. I. 192
Loneragan, J. F.—
  Calcium in the Nitrogen Metabolism of Subterranean Clover 26
Lyne, A. G., and Heideman, Margaret J.—
  The Pre-natal Development of Skin and Hair in Cattle (*Bos taurus* L.) 72

May, L. H., and Buttrose, M. S.—
  Physiology of Cereal Grain. II. Starch Granule Formation in the Developing Barley Kernel .. 146
May, L. H.—
  See also Buttrose, M. S. .. 40
Medicago tribuloides Desr., Effects of Delaying Inoculation on the Seedling Symbiosis of 427
Metabolic Inhibitors, Residual Effects of, in Cell Extension .. 373

PAGE
Millerd, Adele—
  See Hatch, M. D. 167
Molyneux, G. S.—
  The Digestion of Wool by a Keratinolytic Bacillus 274
Moran, P. A. P.—
  Theory of some Genetical Effects of Population Subdivision .. 109
Moran, P. A. P., and Watterson, G. A.—
  The Genetic Effects of Family Structure in Natural Populations 1
Mouse, Variation of Vibrissa Number in the 331
Muir, R. M.—
  See Paleg, L. G. 340
Mushin, Rose, Naylor, Jill, and Lahovary, N.—
  Studies on Plant Pathogenic Bacteria. I, II 223, 233

Nay, T.—
  See Fraser, A. S. 331
Naylor, Jill—
  See Mushin, Rose .. 223, 233
Ng, E. K.—
  See Carr, D. J. 373
Nicholson, R. I., and Lilienthal B.–
  Formation of a Polysaccharide in Sugar-cane 192
Nitella, Studies of Action Potentials in the Vacuole and Cytoplasm of 412
Nitrate Reductase from Germinating Wheat, a DPNH-specific .. 181
Nitrogen Metabolism of Subterranean Clover, Calcium in the 26
Nodulation Studies in Legumes. III 427

O'Donnell, I. J., and Thompson, E. O. P.—
  Studies on Oxidized Wool. II. Extraction of Soluble Proteins from Wool Oxidized with Performic Acid 294

PAGE

O'Donnell, I. J.—
See also Thompson, E. O. P. 282, 490
Ovenden, Stella L.—
See Thaine, R. 349

Paleg, L. G., and Muir, R. M.—
Neutral and Acidic Auxins in Developing Tobacco Fruit .. 340
Pate, J. S.—
See Dart, P. J. 427
Payne, E.—
See Dunstone, J. R. 466
Pearson, Judith A.—
See Hatch, M. D. 167
Physiology of Cereal Grain. I, II 40, 146
Plant Pathogenic Bacteria, Studies on. I, II 223, 233
Plomley, N. J. B.—
Formation of the Colony in the Fungus *Chaetomium* 53
*cyclo*Propane Derivatives, Extension Growth Activities of some 257

Random Mating Populations of Constant Size and Sex Ratio, Genetic Sampling in 500
Rendel, J. M.—
Variation and Dominance at the Scute Locus in *Drosophila melanogaster* 524
Rennin, Action of, on Casein .. 479
Robertson, R. N.—
See Hatch, M. D. 167
Root Initials, Potentiation of Lateral Root Induction by, in Isolated Flax Roots 388

Scute Locus in *Drosophila melanogaster*, Variation and Dominance at the 524
Selection for an Invariant Character, Vibrissa Number, in the House Mouse 506
Setchell, B. P.—
See Lascelles, A. K. 455
Sheep, Hypothyroidism in the .. 455

PAGE

Soil Moisture Regime, Influence of, on the Expulsion of the Larval Mass of the Nematode *Anguina agrostis* from Galls 247
Soybean, Translocation of Labelled Assimilates in the 349
Spencer, D.—
A DPNH-specific Nitrate Reductase from Germinating Wheat 181
Subterranean Clover, Calcium in the Nitrogen Metabolism of .. 26
Sugar-cane, Formation of a Polysaccharide in 192
S-Sulphokerateines and *S*-Carboxymethylkerateines from Wool, Electrophoretic Properties of .. 96

Thaine, R., Ovenden, Stella L., and Turner, J. S.—
Translocation of Labelled Assimilates in the Soybean 349
Thompson, E. O. P.—
A Method for the Isolation of DNP-Cysteic Acid and its Application to the Determination of *N*-Terminal Half-cystine Residues in Wool 303
Thompson, E. O. P.—
See also O'Donnell, I. J. .. 294
Thompson, E. O. P., and O'Donnell, I. J.—
Studies on Oxidized Wool. I. A Comparison of the Completeness of Oxidation with Peracetic and Performic Acids 282
III. Ion-exchange and Acid-uptake Characteristics 490
Thyroxine, Use of, in the Bioassay of Growth Hormone 534
Tobacco Fruit, Developing, Neutral and Acidic Auxins in .. 340
Translocation of Chlorine in Citrus Cuttings during and after a Short Salt Treatment, Uptake and .. 16

PAGE

Translocation of Labelled Assimilates in the Soybean 349

Turner, J. S.—
  See Thaine, R. 349

Vibrissa Number, an Invariant Character in the House Mouse, Selection for 506

Vibrissa Number, Variation of, in the Mouse 331

Wake, R. G.—
  Studies of Casein. IV. The Isolation of κ-Casein 538
  V. Action of Rennin on Casein 479

Wallace, A. L.—
  The Use of Thyroxine in the Bioassay of Growth Hormone .. 534

Watterson, G. A.—
  See Moran, P. A. P. 1

Wheat, Germinating, a DPNH-specific Nitrate Reductase from 181

PAGE

Woods, E. F.—
  The Electrophoretic Properties of *S*-Carboxymethylkerateines and *S*-Sulphokerateines from Wool 96

Wool, Comparison of the Completeness of Oxidation of, with Peracetic and Performic Acids 282

Wool, Digestion of, by a Keratinolytic Bacillus 274

Wool, Electrophoretic Properties of *S*-Carboxymethylkerateines and *S*-Sulphokerateines from .. 96

Wool, Oxidized, Ion-exchange and Acid-uptake Characteristics .. 490

Wool, Oxidized, Studies on. I, II, III 282, 294, 490

Wool Oxidized with Performic Acid, Extraction of Soluble Proteins from 294

Wool, *N*-Terminal Half-cystine Residues in, Method for the Isolation of DNP-cysteic Acid and its Application to the Determination of 303

485